

IMMUNE CONTROL OF EPSTEIN-BARR VIRUS
INFECTION IN AFRICAN CHILDREN

By

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ABSTRACT

Epstein-Barr virus (EBV) establishes a chronic infection, usually effectively controlled by the cellular immune response. However, EBV has the potential to escape immune control, such as during malaria exposure, or modulate the immune response. In this study, immune control of EBV was examined in Gambian children in two situations: during malaria exposure and early after EBV infection. Additionally, EBV infection may also inhibit vaccine induced antibody responses, hence its impact on a childhood pentavalent vaccine was studied, but infection had no effect. In contrast to historical studies, acute malaria infection was not associated with impaired immunity to EBV, a finding potentially explained by the declining malaria exposure in The Gambia. Children recently infected with EBV had evidence of activated EBV-specific T-cell responses, with latent and lytic epitope-specific responses of equal magnitude. Several donors identified as undergoing primary asymptomatic EBV infection had virus genome loads equivalent to those of acute infectious mononucleosis (AIM) patients. In contrast to AIM patients they did not show a peripheral lymphocytosis but did have significant expansions of activated EBV-specific CD8⁺ T-cells, which were lower or perhaps more focused than in AIM patients, suggesting that the highly expanded T-cell populations and not virus load drives AIM pathogenesis.

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LIST OF ABBREVIATIONS

ACV	Acyclovir
AIDS	Acquired immunodeficiency syndrome
AIDS-BL	Acquired immunodeficiency syndrome associated Burkitt lymphoma
AIM	Acute infectious mononucleosis
anti-HBs	Hepatitis B surface antibody
APC	Allophycocyanin
BamA	BamA rightward transcript
Bcl-2	B-cell lymphoma 2
BSA	Bovine serum albumin
CD	Cluster of differentiation
CIDR1 α	Cysteine-rich inter-domain region 1 alpha
CMV	Cytomegalovirus
CSA	Cyclosporine A
CTL	Cytotoxic T lymphocyte
CY	Cascade yellow
Cy	Cyanine dye
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DTP	Diphtheria, Tetanus and <i>Bordetella pertussis</i>
Dtx	Diphtheria toxin
E	Early
EBER	Epstein-Barr Virus -encoded Ribonucleic Acid
eBL	Endemic Burkitt Lymphoma
EBNA	Epstein-Barr Virus nuclear antigen
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot assay
EPI	Expanded programme for immunisation
FACS	Fluorescent-associated cell sorting
FBC	Full blood count
FCS	Fetal calf serum
FHA	Filamentous Haemagglutinin
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FPLC	Fast protein liquid chromatography
FSC	Forward scatter
GC	Germinal centre
gp	Glycoprotein
HBsAg	Hepatitis B surface antigen
Hcl	Hydrochloric acid

HepB	Hepatitis B
HHV	Human Herpes Virus
Hib	<i>Haemophilus influenzae b</i>
HIV-1	Human immunodeficiency virus type 1
HLA	Human leukocyte antigen
HPF	High power fields
HRP	Horseradish peroxidase
ICS	Intracellular cytokine staining
IE	Immediate early
IFN- γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
kDa	Kilodalton
L	Latency membrane protein
LCL	Lymphoblastoid cell lines
LCV	Lymphocryptovirus
LMP	Latency membrane protein
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
MHC	Major Histocompatibility Complex
MIP1 β	Macrophage inflammatory protein 1 beta
MRC	Medical Research Council
MSP	Merozoite surface protein
NK	Natural killer
NPC	Nasopharyngeal Carcinoma
OD	Optical density
OPD	O-phenylenediamine dihydrochloride
<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
PAMPs	Pathogen-associated molecular intracellular patterns
PB	Pacific blue
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
PHA	Phytohaemagglutinin
pLDH	Parasite lactate dehydrogenase
POL	Polymerase
Prn	Pertactin
PTLD	Post-transplant lymphoproliferative disorder
Ptx	Pertussis toxin
RNA	Ribonucleic Acid
RSV	Respiratory syncytial virus

sBL	Sporadic Burkitt lymphoma
SEB	<i>Staphylococcus</i> Enterotoxin B
SFC	Spot forming cells
SSC	Side scatter
SSP	Sequence specific primers
T-bet	T-box transcription factor
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
TLR	Toll- like receptors
TNF- α	Tumour necrosis alpha
Ttx	Tetanus toxin
<i>uv</i>	<i>Ultraviolet</i>
VCA	Viral capsid antigen
WIMM	Weatherall Institute for Molecular Medicine

CHAPTER 1 : INTRODUCTION

1.1 The development and survival of lymphocytes

The adaptive immune response enables an individual to make immune responses against a wide variety of pathogens during their lifetime. Diverse repertoires of T- and B-cell receptors are generated during T- and B-cell development in central lymphoid tissues. Both T- and B-cell lymphocyte precursors originate in the bone marrow, B-cells complete most of their development there whereas the majority of T-cells migrate to the thymus.

Within the specialised microenvironment of the bone marrow signals from stromal cells act on the lymphocyte progenitors allowing subsequent differentiation of B-cells. The heavy chain locus is rearranged first and if successful combines with surrogate light chains. These undergo rearrangement and if productive form a complete immunoglobulin B-cell receptor. Immature B-cells are tested for autoreactivity before they leave the bone marrow. Cells that have no strong reactivity to self antigens are allowed to mature however if they are self reactive their development is arrested and the cell will not mature. There are several possible fates for self-reactive B-cells; cell death by apoptosis (“clonal deletion”); the production of a new receptor by a process known as receptor editing, the induction of a permanent state of unresponsiveness or anergy to antigen and immunological ignorance.

T-cell progenitors derive from pluripotent haematopoietic stem cells in the bone marrow. They then migrate to the thymus where they mature. Similar to B-cells T-cells rearrange antigen-receptor genes. Two distinct lineages are generated $\gamma:\sigma$ and $\alpha:\beta$ expressing distinct antigen-receptor genes. On migration to the thymus T-cell precursors spend up to a

week differentiating before proliferating. T-cells undergo marked changes in T-cell receptor expression. Surface proteins are used as markers of the stage of T-cell differentiation with the $\alpha:\beta$ T-cells forming two distinct functional subsets CD4 and CD8. Only immature T-cells whose receptors interact with self-peptide: self-MHC (major histocompatibility complex) complexes survive, the others die in the thymus. The CDR1 (complementary-determining regions) and CDR2 loops of both chains of the T-cell receptor give the T-cell receptor an intrinsic specificity for the MHC molecules. T-cells initially express both CD4 and CD8 T-cells but by the end of thymic selection express only one of these co-receptors. Almost all mature T-cells that express CD4 recognise peptides bound to self-MHC Class II molecules and the majority are programmed to become cytokine-secreting cells. In contrast, the majority of cells expressing CD8 have receptors that recognise peptides bound to self-MHC Class I molecules and are programmed to have cytotoxic function. T-cells that react strongly to ubiquitous self-antigens are deleted in the thymus through negative selection. Peripheral tolerance also occurs where by self-reactive lymphocytes in the periphery are eliminated through deletion, anergy or ignorance.

MHC Class I molecules are found on the surface of cells consisting of a trimeric complex of beta-2-microglobulin (β_2M), MHC heavy chain and peptide. The formation of mature MHC Class I complexes involves targeting intracellular proteins for degradation. Proteins are cleaved into short peptide fragments which are transported into the endoplasmic reticulum (ER) lumen loaded onto MHC class I molecules to form a stable complex. The peptide-loaded class I molecules are exported to the Golgi where they are glycosylated and trafficked to the cell surface. The main pathway of protein degradation

within cells is via the proteasome, a multicatalytic proteinase complex abundant in all cells except erythrocytes. Proteasomes exist as either a 20S or 26S complex. The translocation of peptides into the ER is mediated predominantly by TAP (transporters associated with antigen processing).

Naïve B-cells can be activated in T-cell dependent and independent manners. The majority require help from CD4⁺ T helper-cells to become activated. This occurs in secondary lymphoid tissue (lymph nodes, spleen and mucosal sites such as the gut and respiratory tract) where Germinal Centres (GC) form. Antigen cross-links the B-cell receptor and then additional cognate interaction with antigen-specific helper T-cells (Th2 cells) occurs. The primed T-cell then secretes cytokines that activate the B-cell. B-cells then proliferate and differentiate into long-lived plasma cells that synthesise and secrete vast amounts of antibody. The first antibodies made are always IgM and IgD. By contact with effector T-helper cells activated B-cells can undergo class switching of their heavy chain isotype to produce IgG, IgA and IgE and usually no-longer make earlier isotypes of IgM or IgD. T-cell independent activation can occur when B-cells receive secondary activation through toll-like receptors or the antigen is a molecule able to simultaneously cross-link enough B-cell receptors to activate the cell.

CD8⁺ T-cells play a critical role in human responses against viruses. The majority of our knowledge about antigen induced CD8⁺ T-cells comes from the study of immune responses in mice. Viral infection drives clonal expansion of antigen-specific T-cells and the acquisition of effector functions leading to elimination of infected cells. Contraction of the expanded pool of cells occurs following neutralisation of the pathogen with only

10% of cells persisting as memory cells (Murali-Krishna et al., 1998). How these memory cells develop remains incompletely understood. A divergent model suggests that these cells are formed early in the response against infection and persist as the acute effectors die out. Whereas the linear differentiation model suggests that they develop from effector cells as the antigen load drops (Kaech et al., 2002). Multiple cell surface phenotypes in humans have been proposed as markers of T-cell differentiation; largely combinations of isotypes of CD45R and receptors involving activation, co-stimulation and lymphocyte homing. Rene van Lier originally described that T-cells can be divided into naïve, effector and memory subsets using CD45RA and CD27 (Hamann et al., 1997). Another descriptive phenotype proposed uses CCR7 (chemokine receptor 7) expressing T-cells as they have the ability to migrate to lymph nodes (Sallusto et al., 1999). CD45RA-CCR7+ T-cells have been termed central memory cells, with CD45RA-CCR7- cells termed peripheral effector memory as they migrate to peripheral tissue and have a greater ability to produce effector cytokines than central memory cells (Sallusto et al., 1999). The existence of these functional difference has however been questioned and CD45RA-CCR7- T-cells can be found in lymph nodes. Furthermore, although naïve CD8+ T-cells express CD45RA, primed cells have also been shown to express CD45RA, therefore its expression is not specific to naïve cells. Victor Appay has proposed a description of virus specific cells that does not use CD45RA expression describing them as being on a pathway of differentiation based on expression of CD27 and CD28 progressing from early to intermediate to late differentiation (Appay et al., 2002a). For the majority of these markers expression alters during T-cell development for example CCR7 and CD28 expression up-regulation on activation (van Leeuwen et al., 2002,

Wierenga et al., 1991). A causal link between phenotype and function for most markers in humans remains ambiguous and is the subject of current debate (Appay et al., 2008).

In the majority of acute viral infections in humans CD8 T-cells, through MHC Class I tetramer staining, have been shown to be activated and proliferating as evidenced by expression of CD38, HLADR and Ki-67, a nuclear antigen marker of cell cycling. Most express CD45RO, with few expressing CCR7 (Catalina et al., 2002, Gamadia et al., 2003, Urbani et al., 2002, Wills et al., 2002) however during recovery there is increased expression of CD45RA on antigen-specific cells in the case of EBV and CMV infections (Callan et al., 1998b, Gamadia et al., 2003, Wills et al., 2002). Early on in the acute response to EBV, CMV and HIV, CD28 is expressed on antigen-specific CD8 T-cells with expression reducing over time (Appay et al., 2002b, Callan et al., 1998a, Gamadia et al., 2003, Roos et al., 2000).

1.2 Biology of Epstein-Barr virus

Epstein-Barr virus (EBV) is one of eight known human herpesviruses, designated human herpesvirus 4 (HHV-4) and is a member of the gamma herpesvirus family. All gamma herpesviruses infect lymphoid cells, using these to establish the site of latency and also replicate in certain other cell types. For EBV the major host cells are B-lymphocytes, although it is capable of lytic replication in oropharyngeal epithelial cells (Greenspan et al., 1985, Birkenbach et al., 1992, Li et al., 1992, Sixbey et al., 1983).

EBV is a linear double stranded DNA virus (172-kilobase-pair) encoding approximately 100 genes and was completely sequenced in the early 1980s (Baer et al., 1984). It has a toroid-

shaped protein core and an icosahedral nucleocapsid (major capsid proteins range in size from 28-160 kDa). A protein tegument surrounds the capsid and surrounding this is an outer envelope containing at least eight glycoproteins (Hutt-Fletcher, 2007, Dolyniuk et al., 1976, Roizman et al., 1993, Thorley-Lawson and Edson, 1979).

Initial infection in the majority of humans occurs in the oropharynx via saliva transmission of the virus. The first cellular target of EBV infection, be it epithelial or B-cell, is unclear, however the virus will infect B-cells via the virus glycoprotein gp350/ B-cell surface molecule CD21 (CR2, complement receptor 2) interaction (Ahearn et al., 1988, Nemerow et al., 1987, Nemerow et al., 1986, Tanner et al., 1987). Further glycoproteins are involved in viral attachment and entry, amongst which are gp42, which binds the human leukocyte antigen (HLA) class II cell surface molecules of B-cells and gp85, which allows fusion of the viral envelope with the cell membrane. The fusion step releases the nucleocapsid into the cell cytoplasm where it subsequently dissolves (Haddad and Hutt-Fletcher, 1989, Miller and Hutt-Fletcher, 1988, Wang and Hutt-Fletcher, 1998, Adams, 1987). The EBV genome is then transported to the nucleus where its replication can occur using cellular DNA polymerases during the cell cycle S phase (Adams, 1987).

When the viral genome enters the nucleus a subset of genes may be expressed known as latency genes, the products of which endow EBV with growth-transforming ability for its principal target cell, the B lymphocyte, and ensure transfer of the genome to the daughter cells (Babcock et al., 2000, Miyashita et al., 1997). Thus, EBV colonises the B-cell system via a transient growth-transforming infection and then persists by down regulating expression of the growth transforming latency genes to establish an antigen-negative form of latent

infection in which the genome sits in the nucleus in a mostly quiescent state (Fields et al., 2007). The cells in which EBV resides appear phenotypically as memory B-cells and these preferentially circulate between the blood and oropharyngeal lymphoid tissue, collectively known as Waldeyers ring, and the virus may reactivate at this site into its virion productive or lytic cycle (see below) (Hadinoto et al., 2009).

EBV persists through the establishment of latent infection, a state of viral persistence with a lack of viral replication, although ability to reactivate and replicate is retained. The majority of viral DNA in latently infected cells is in a circular form (Hurley et al., 1991, Matsuo et al., 1984). Latency allows the virus to evade host immune responses by limiting viral gene expression to the noncoding EBV-encoded RNA (EBER) and BamA rightward transcript (BART) RNAs, with no expression of protein-coding transcripts, which is sometimes termed latency 0 (Fields et al., 2007). The virus can reactivate from this state into the host cell growth transforming infection by expressing the latency genes, namely the six nuclear (EBNAs) and membrane proteins (LMP1 and LMP2) (Klein et al., 2007). However, EBV can adopt several latency programmes, which express characteristic sets of these genes, mostly described in relation to the clinical manifestations the virus causes. The full range of latent gene expression, seen in the growth transforming infection, also known as latency III, is observed in tonsillar B-cells during infectious mononucleosis (Laytragoon-Lewin et al., 1997), in posttransplant lymphoproliferative disease (PTLD) (Young et al., 1991) and also in B-cell cultures infected with EBV *in vitro*, also known as B-lymphoblastoid cell lines (LCLs) (Rowe et al., 1992). Other latency forms are seen in certain EBV-associated disease states: latency I where only EBNA1 is expressed is seen in the B-cell malignancy eBL, while latency II gene expression patterns in which EBNA1, LMP1 and LMP2 are expressed are seen in the B-cell

malignancy Hodgkin lymphoma and the epithelial malignancy nasopharyngeal carcinoma (NPC) (Table 1.1).

Table 1-1 *EBV associated malignancies and latency patterns*

Malignancy	% EBV association (distribution)	Patterns of latent gene expression
Burkitt B-cell lymphoma	Endemic 95% (Central Africa) Sporadic 10% (Worldwide) AIDS-associated 30%	Type I EBNA1 expression (Subset express LMP1 and EBNA2)
Nasopharyngeal Carcinoma	90% (China and South East Asia)	Type II EBNA1 with variable levels of LMP proteins
Hodgkin lymphoma	40% (Worldwide)	Type II High level of LMP1 and no EBNA2 expression
Post-transplant Lymphoproliferative disorder (PTLD)	90% (Solid organ transplant recipients)	Type III Full-set of latency genes expressed

EBV is thought to frequently spontaneously reactivate into lytic cycle *in vivo* (Prang et al., 1997, Tan et al., 1999) with expression of more than 80 viral genes. Identification of lytically infected cells has not been definitively demonstrated in healthy infected individuals, but virus has been detected in throat washings and persistence of antibodies to lytic cycle antigens suggest that there is reactivation in lytic cycle occurring in healthy carriers. What specifically triggers reactivation is yet to be elucidated, however Laichalk *et al.* have demonstrated that differentiation into plasma cells initiates EBV replication, but whether this leads to the production of infectious virus could not be determined (Laichalk and Thorley-Lawson, 2005). Antigen-driven plasmacytoid differentiation is therefore one possible trigger for reactivation and lytic cycle expressed proteins have been identified in rare plasma cells in tonsillar sections (Shaknovich et al., 2006).

During the lytic cycle there are three time dependent phases of viral lytic gene expression: immediate-early (IE), early (E) and late (L). The two IE proteins, BZLF1 and BRLF1, act as transcriptional transactivators for other lytic cycle genes. The second wave of genes expressed encoding E proteins have a wide range of functions including DNA replication, metabolism and blockage of antigen processing, whereas genes expressed in the late stage of lytic infection are mostly structural viral proteins such as VCA (Tsurumi et al., 2005). Potentially then, many gene products exist which the immune system can target.

1.3 Epidemiology of Epstein-Barr virus

EBV is a gamma herpesvirus estimated to infect more than 90% of the human adult population worldwide (Evans et al., 1968). There are two closely related subtypes of EBV, EBV-1 and EBV-2, with extensive homology throughout most of their genomes (Bornkamm et al., 1980, Sample et al., 1990, Zimmer et al., 1986). The major differences that have been identified are between the latent infection EBNA genes, EBNA-2, EBNA-LP, EBNA-3A, -3B and -3C, with the relevant EBNA proteins sharing between 55-85% primary amino acid sequence identity, EBNA-2 being the most divergent (Sample et al., 1990, Raab-Traub and Flynn, 1986). The subtypes have been noted to have differing transforming capacities and altered abilities to spontaneously enter the lytic cycle, with type 2 viruses being less efficient transformers (Rickinson et al., 1987) and their derived cell lines slightly more permissive for virus replication (Buck et al., 1999). Sero-epidemiological studies have determined that in the United States and Europe, type 1 strains are the predominant circulating viruses, but that the two subtypes are more equally distributed in Africa (Nicholas et al., 1992, Zimmer et al., 1986).

EBV is associated with occasional severe self-limiting primary infections, but it does not cause significant morbidity in the majority of immunocompetent infected individuals. However EBV is an important pathogen associated with several malignancies. EBV was the first isolated human tumour virus (Epstein et al., 1964), and is also the cause of significant pathology in immunosuppressed hosts.

In developing countries, such as The Gambia, high rates of EBV infection are seen in infancy, in contrast to most developed countries where the majority of primary infection occurs at a much later stage, often in adolescence (Biggar et al., 1978a, Miles et al., 2007, Njie et al., 2009, Balfour et al., 2013). EBV infection is most likely to be acquired in young children through close contact involving exchange of oral secretions (Hoagland, 1955), which is likely to be increased in multiple occupancy households such as is seen in developing countries, where overcrowding is common. EBV infection has also been acquired iatrogenically from peripheral blood (Gerber et al., 1969), transplanted haematopoietic cells (Alfieri et al., 1996, Shapiro et al., 1988) and solid organ transplant (Hanto et al., 1981). EBV naïve patients who receive a donor organ from an EBV-infected donor are at high risk for developing PTLT (Knight et al., 2009). Transmission through sexual intercourse has also been suggested and EBV has been recovered from male and female genital tracts (Crawford et al., 2006, Higgins et al., 2007, Enbom et al., 2001, Naher et al., 1992, Sixbey et al., 1986). Kissing and sexual intercourse are, however, virtually always carried out simultaneously, so it is difficult to completely exclude oral transmission in these cases, and more recently, transmission has been shown not to differ in subjects reporting deep kissing with or without penetrative sexual intercourse (Balfour et al., 2013).

1.4 B-cells and Epstein-Barr virus

Naïve B-cells given CD4⁺ T-cell help proliferate in the spleen and lymph nodes and once activated can continue down one of two pathways, either remaining static and differentiating into short-lived plasma cells or migrating into B-cell follicles and initiating a GC reaction (Gourley et al., 2004, MacLennan, 1994, McHeyzer-Williams et al., 2001). During the GC reaction, B-cells generate diversity through somatic hypermutation of immunoglobulin genes to create a spectrum of cells with individual antibody genes that have different affinities for the antigen. These cells undergo clonal selection in which B-cells with the highest affinity receptors for the antigen are selected to produce antibody and form memory B-cells. (Leanderson et al., 1992, MacLennan, 1994, McHeyzer-Williams et al., 2001, Tarlinton and Smith, 2000, Ziegner et al., 1994). Ultimately this results in the production of high affinity memory B-cells. A common feature of somatically mutated B-cells subsets is the expression of the CD27 cell surface antigen and this is commonly regarded as a memory marker for B-cells (Klein et al., 1998). Memory B-cells persist for decades after the initial antigen exposure through infection or vaccination. Periodic re-exposure boosts memory B-cell numbers be it through re-infection, chronic low-grade infection or a vaccination booster. However, memory B-cells and antibody levels can be maintained for years in the absence of antigen re-exposure (Crotty et al., 2003), but how this occurs remains to be elucidated.

EBV establishes latency in the memory B-cell pool. In healthy carriers, between 1 in 10⁴ and 1 in 10⁶ of memory B-cells are infected with EBV, however during acute infectious mononucleosis (AIM) it has been shown that as much as 50% of the memory B-cell pool harbours EBV latent virus (Babcock et al., 2000, Hochberg et al., 2004). As EBV infects both memory and naïve B-cells equally *in vitro* (Ehlin-Henriksson et al., 2003), how the virus

becomes restricted to the memory B-cell pool *in vivo* is a subject of much debate. One model suggests that EBV initially infects naïve B-cells, and by mimicking antigen stimulation utilises the physiological B cell germinal centre (GC) reaction to drive the B-cell into a memory cell (Thorley-Lawson, 2001, Thorley-Lawson and Babcock, 1999). Others dispute this model, as EBV has been isolated from non-isotype switched (IgD+CD27+) memory cells, that evidence suggests are GC independent in origin (Chaganti et al., 2009). They therefore favour the hypothesis that pre-existing memory B-cells are preferentially infected or that the virus infects both naïve and memory B-cells but the latter have a survival advantage (Chaganti et al., 2009, Kuppers, 2003).

There have been few studies looking at the impact of EBV infection on total B-cell subsets and their functionality. Souza *et al.* looked at peripheral blood memory B-cells in AIM patients infected with EBV and compared them to uninfected B-cells. The EBV positive cells were found to accumulate more somatic hypermutations than EBV negative cells. EBV was also found to occupy a skewed niche within the memory compartment due to its exclusion from the IgM+IgD+CD27+ B-cell subset (Souza et al., 2007). Heath *et al.* demonstrated similar findings *in vitro*, with EBV infected B-cells displaying immunoglobulin heavy chain sequence changes similar to somatic hypermutations (Heath et al., 2012).

1.5 Innate Immunity, Immune Evasion and Epstein-Barr virus

The first line of defence against viral infections is provided by the innate immune system. Toll-like receptors (TLRs) are responsible for the recognition of specific pathogen-associated molecular intracellular patterns (PAMPs) expressed on viruses and other microorganisms. They act via multiple pathways, such as activation of the NF- κ B pathway, to link the innate

and adaptive immune response. Recent literature has demonstrated the involvement of several TLRs (TLR2 (Ariza et al., 2009), TLR3 (Iwakiri et al., 2009), TLR7 and TLR9 (Quan et al., 2010)) in response to EBV infection. Further recent studies have suggested that EBV modulates TLR signalling pathways as an immune evasion strategy (van Gent et al., 2011, Younesi et al., 2010). In an *in vitro* model EBV down regulates TLR9 expression through RNA degradation and a further study demonstrates significant inhibition of proliferative responses to TLR7/8 and TLR9 agonists (van Gent et al., 2011, Younesi et al., 2010).

The importance of host T-cell responses in maintaining an asymptomatic virus-host balance is clinically evident for EBV. In the West, T-cell-suppressed transplant patients are more vulnerable to potentially lethal EBV-driven B-cell expansions presenting as PTL (Rezk and Weiss, 2007, Rickinson, 2002), and human immunodeficiency virus (HIV-1) infected patients experience distinct lymphoproliferative diseases (Long and Sample, 2007). A large range of lytic cycle proteins have had CD8⁺ responses identified through screening of AIM derived clones. Responses have been found to focus on two IE proteins, BZLF1 and BRLF1 with smaller sub dominant responses to the following E proteins; BMLF1, BMRF1, BALF2 or BALF5. Clones reactive to other E or L proteins are a minor component of the CD8⁺ response (Hislop et al., 2002, Pudney et al., 2005, Steven et al., 1997). As cells progress through the lytic cycle, their HLA class I antigen-processing capacity becomes impaired through the action of several viral proteins. For example, the EBV lytic cycle protein BNLF2a has been shown to block class I antigen presentation through inactivating the TAP1/TAP2 peptide transporter as possible mechanism for the aforementioned impairment in processing (Croft et al., 2009, Hislop et al., 2007a). Despite potent T-cell responses during acute infection, EBV is able to evade the immune response and persist by employing such immune

evasion strategies (Bell et al., 2009, Horst et al., 2009, Thorley-Lawson, 2001, Thorley-Lawson and Gross, 2004).

1.6 Primary Epstein-Barr virus infection: Acute infectious mononucleosis and asymptomatic primary infection

Glandular fever was initially described in 1889 and later termed AIM in 1920 when describing six college students with a febrile illness and an atypical lymphocytosis (Evans, 1974, Sprunt TP, 1920). Most studies suggest about a quarter of individuals undergoing primary EBV infection in adolescence will develop a sometimes severe disease, AIM (Crawford et al., 2006). In contrast, those infected in childhood either undergo an asymptomatic infection or have mild self-limiting illnesses, which largely go unrecognised; however rare instances of AIM in infancy have been reported (Chan et al., 2003, Crawford et al., 2006, Sumaya et al., 1975). More recently however, it has been suggested that far more adolescents than previously thought have AIM at the time of primary EBV infection (Balfour et al., 2013). However, this should be interpreted with caution since this study classified AIM symptomatic patients as having at least two out of the four following symptoms: sore throat, cervical lymphadenopathy, fever and fatigue, which are by no means pathognomonic for EBV. In addition they provided intensive follow-up on an eight weekly basis to screen for these symptoms, however the number of participants showing these symptoms who did not have evidence of serological primary EBV infection was not reported. Thus, other intercurrent infections such as cytomegalovirus (CMV), respiratory syncytial virus (RSV), adenovirus or streptococcal pharyngitis that are also common may also account for the quartet of symptoms they described (Balfour et al., 2013).

Typically AIM patients have a prodromal phase of malaise and headache with a mild fever prior to developing more specific symptoms and signs such as a pharyngitis, lymphadenopathy and an atypical lymphocytosis (Evans, 1989, Luzuriaga and Sullivan, 2010, Peter and Ray, 1998). A review of over 500 patients found that lymphadenopathy was present in all patients, fevers in 98% and pharyngitis in 85% (Hoagland, 1975, Rea et al., 2001). Interestingly, the very young or older adults often do not develop the classical clinical picture, with those aged 40-70yrs more frequently complaining of pharyngitis and myalgia and cervical lymphadenopathy being less common (Auwaerter, 1999, Horwitz et al., 1983). Other clinical manifestations can be seen such as splenomegaly (50-60% of patients) (Carter and Penman, 1969), hepatitis e.g. abnormal liver function tests (80% of patients) (Odumade et al., 2011) and more rarely a generalised maculopapular, urticarial or petechial rash, Guillain-Barré syndrome, facial or cranial nerve palsies (Johns and Hogikyan, 2000, Joki-Erkkila et al., 2000, Long and Kerschner, 2001), meningoencephalitis (Schellinger et al., 1999), aseptic meningitis, transverse myelitis, peripheral neuritis, optic neuritis and encephalomyelitis (Tselis et al., 1997). Recovery from AIM is gradual and although the median duration of illness is 16 days it can sometimes take months for an individual to feel entirely well with persistence of fatigue often being a prominent symptom (Rea et al., 2001).

In primary symptomatic EBV infection, it is estimated that from the initial infection there is a period of five to seven weeks before a patient will develop AIM (Svedmyr et al., 1984). This lag time means that patients do not often present during the incubation period. AIM patients' viral loads in the oral cavity, (oral cells and saliva) are higher than those found in peripheral blood or PBMCs and remain elevated for months (Balfour et al., 2005, Fafi-Kremer et al., 2005, Hadinoto et al., 2008), whereas the latter are reduced more rapidly to levels typical of

healthy long-term virus carriers usually by week seven of illness (Balfour et al., 2005, Balfour et al., 2013).

Another important arm of the innate immune system are natural killer (NK) cells. Expansions of NK cell numbers have been demonstrated during AIM and numbers inversely correlated with peripheral blood viral load (Williams et al., 2005) suggesting that NK cells could play a role in limiting viral replication. However, more recently the converse has been shown and NK cells numbers have been found to positively correlate with peripheral blood viral load and disease severity (Balfour et al., 2013). In addition the clinical evidence does not support the former theory, as recipients of T-cell depleted stem cell transplants commonly develop EBV driven PTLD in the first 3-6 months post-transplant at which point NK cells numbers have recovered but the patients are still T-cell deficient (O'Reilly et al., 1997, Hislop et al., 2007b).

The antibody response to EBV is used in the diagnosis of AIM. In a recent study, 90% of patients with primary EBV infection had detectable levels of IgM antibody against the viral capsid (anti-VCA IgM) within seven days of onset of symptoms (Balfour et al., 2013). Following this they all subsequently developed anti-VCA IgG antibodies, which then persisted for life. IgM anti-VCA antibodies decline to lower levels and disappear within one to two months (Epstein and Achong, 1979, Fleisher et al., 1979). In terms of latent antigen responses, an anti-EBNA-2 response is regularly detectable in AIM whereas anti-EBNA-1 antibodies are unusually delayed. This has recently been shown to correlate with the slow emergence of EBNA1-specific CD4⁺ T-cells compared to CD4⁺ T-cells specific to epitopes from other EBNA proteins in AIM patients (Long *et al* J Exp Med, *in press*). The heterophile antibody response, a cross-reactive antibody response, is seen in over 90% of adults with AIM

(Horwitz et al., 1975). However, this lacks sensitivity in childhood cases, as heterophile antibody responses are either minimal or not detectable (Epstein and Achong, 1979, Biggar et al., 1978a, Biggar et al., 1978b).

Many studies over the years have characterised cellular immune responses, both to CD8+ and less so to CD4+ T-cells, during primary infections (AIM) in adults (Amyes et al., 2003, Callan et al., 1996, Callan et al., 1998b, Catalina et al., 2001, Faint et al., 2001, Hislop et al., 2002, Hislop et al., 2005, Hislop et al., 2007b, Hoshino et al., 1999, Pudney et al., 2005, Steven et al., 1997, Steven et al., 1996, Woodberry et al., 2005). Individual lytic epitope responses seen in AIM can account for 1-40% of the total CD8+ T-Cell population. Responses to IE and E proteins of the lytic cycle usually dominate. The latent cycle protein responses tend to be smaller, accounting for only 0.1-5% of the CD8+ T-cell population. Typically they focus on epitopes derived from the EBNA3 family of proteins, namely 3A, 3B and 3C. In these donors EBV-specific CD8+ T-cells have been well described as displaying a phenotype consistent with recent antigen stimulation. Thus, they are perforin positive with direct *ex vivo* cytotoxic function (Callan et al., 2000, Steven et al., 1997, Steven et al., 1996), express activation markers such as CD38 and cell cycling marker Ki-67 (Callan et al., 1998b, Catalina et al., 2001, Hislop et al., 2002, Hislop et al., 2005). These cells die rapidly *in vitro* unless given an antigen stimulus or cytokines which signal through the common γ -chain receptor, since they express high levels of the pro-apoptotic protein Bax and low levels of the anti-apoptotic molecule bcl-2 (Callan et al., 2000, Dunne et al., 2002, Tamaru et al., 1993). Consistent with this, *in vivo* CD8+ T-cells are rapidly culled in the weeks post infection, lytic responses declining more rapidly than latent. It is therefore not surprising that quantitative changes are seen when comparing the dominance hierarchies of lytic and latent epitope-

specific responses between persistent infection and AIM (Hislop et al., 2002, Woodberry et al., 2005).

The CD4⁺ T-cell pool is not typically expanded in AIM. EBV-specific CD4⁺ T-cells have been less well characterised in view of the relative lack of well defined epitopes and the difficulties surrounding HLA class II tetramer manufacture. However, as with the greater CD8⁺ responses, EBV-specific CD4⁺ T-cells have been identified during AIM against lytic (BZLF1 and BMLF1) and latent antigens (EBNA1 and EBNA3A) and decline rapidly within weeks (Hislop et al., 2007b, Precopio et al., 2003). A very recent study using EBV MHC class II tetramers has refined these earlier observations, all of which were based on detecting very small CD4⁺ T-cell responses to EBV peptide pools or protein preparations by cytokine-based assays. This shows that actual sizes of CD4 reactivities are five to ten fold underestimated by cytokine-based assays (Long *et al* JEM *in press*).

In contrast to the wealth of immunological and virological data on primary infection in AIM patients, the study of subclinical primary EBV infection has proved challenging due to the logistical difficulties in identification of asymptomatic patients undergoing primary infections. Silins *et al.* fortuitously identified a subset of patients undergoing silent seroconversion in a phase I EBV vaccine trial and were able to demonstrate that in contrast to the large T-cell expansions seen during AIM, asymptomatic donors showed little evidence of alterations in their lymphocyte populations (Silins et al., 2001). This was seen despite equally high EBV genome loads in both AIM and asymptomatic patients. More recently, in a cohort of adolescent university students (median age 18.6 years) followed prospectively, those who developed EBV infection were assessed at eight weekly intervals with intensive follow-up

measuring EBV viral genome loads, CD8 lymphocytosis and NK cell percentage (Balfour et al., 2013). These three markers were found to positively correlate with disease severity, as determined by a laboratory-documented severity of illness score (Balfour, 2007, Balfour et al., 2005). A higher than previously reported proportion of these students, 89%, were reported as having symptomatic AIM, but as noted above they did not report symptom detection rates in patients that were not seroconverting nor did they control for possible confounders such as co-infections. It has been proposed that differing forms of primary EBV infection could be responsible for causing AIM (Silins et al., 2001). It has also been suggested that reactivation of memory CD8⁺ T cells specific to previously encountered pathogens, but cross-reactive with EBV (and therefore a cross-reactive heterologous immune response), contributes to the expansions seen in AIM (Clute et al., 2005). However what precisely dictates whether someone develops AIM or undergoes an asymptomatic primary infection remains elusive.

1.7 Persistent Epstein-Barr virus infection: Life-long carriers

During long-term asymptomatic EBV carriage, individuals often shed much lower levels of virus and have lower numbers of latently infected B-cells in the blood compared to levels seen during AIM. The low levels of persisting virus and intermittent viral reactivation likely maintain persistent low-levels of antigen stimulation. The immune responses in healthy adult carriers with persistent infection and no history of AIM have been previously explored (Amyes et al., 2003, Hislop et al., 2001, Hislop et al., 2007b, Tan et al., 1999). Asymptomatic virus carriers maintain T-cell memory both to latent and lytic cycle antigens. CD8⁺ T-cells specific for lytic cycle antigens generally constitute 0.2 to 2% of the CD8⁺ T-cell population, while the CD4 lytic antigen response is less well studied but again appears to be less numerous than the CD8⁺ response (Hislop et al., 2007b, Leen et al., 2001, Long et al., 2005).

With respect to latent antigen responses, CD8⁺ T-cells specific for known EBV-epitopes are relatively common, ranging from 0.05 to 1% of CD8⁺ T-cells in the blood and are typically ten-fold more numerous than their CD4⁺ counterparts. The CD8⁺ memory T-cells tend to display a resting phenotype, lacking activation markers and expressing much higher levels of bcl-2 than EBV-specific T-cells from AIM patients. There are, however, some that still express perforin and are cytotoxic *ex vivo* (Callan et al., 1998b, Catalina et al., 2002, Dunne et al., 2002, Faint et al., 2001, Hislop et al., 2001). The proportion of EBV-specific CD8⁺ T-cells tends to remain reasonably stable over time with some variation from the mean over months (Crough et al., 2005). As age increases (over years rather than months), there is evidence of expansion of EBV-specific responses, which interestingly is not seen in patients who are co-infected with CMV (Khan et al., 2004, Ouyang et al., 2003).

Recent work has shown that EBV-specific CD8⁺ T-cell populations in asymptomatic carriers are oligoclonal with dominance of a few prevalent clonotypes with high avidity (Price et al., 2005). The peripheral TCR (T-cell receptor) repertoire acts as a reservoir for recruitment of diverse antigen-specific clonotypes with subdominant clonotypes with lower avidities also identified. Some common (public) antigen-specific clonotypes were observed in certain individuals, which differed at the nucleotide level, implying that there is an on-going selective process *in vivo* operating at the protein level. These observations suggest that such processes are present to optimise antiviral effector functions while maintaining clonotypic diversity within the reactive memory T-cell pool (Price et al., 2005).

Differences between the CD4⁺ and CD8⁺ T-cell responses have been identified in EBV-seropositive donors with no history of AIM. Both CD4⁺ and CD8⁺ T-cell populations

broadly target all latent cycle antigens to some degree, although the EBNA-3A, B, C family of antigens elicit the strongest CD8⁺ T-cell responses in most individuals, while the EBNA-1 antigen is the commonest, but by no means the only, target of the CD4⁺ T-cell (Leen et al., 2001, Hislop et al., 2007b). These apparent trends in immunodominance of responses to latent antigens do not bear any relation to the order in which these antigens appear during EBV-induced B-cell transformation, where EBNA-2 and EBNA-LP expression precedes that of the other latent proteins (Fields et al., 2007). This is in contrast to what is seen for lytic CD8⁺ T-cell antigen responses where the hierarchy of immunodominance reflects the antigens' order of appearance during the virus lytic cycle. Thus, IE and some E proteins tend to induce the numerically dominant responses and L proteins induce weaker responses. Interestingly this hierarchy reflects the efficiency with which these proteins are presented on the surface of lytically infected cells, since virus-coded evasion proteins progressively impair the antigen-processing function as the cells move through lytic cycle. The correlation between immunodominance and timing of expression implies that direct interaction with lytically infected cells is the main driver of the expanding CD8⁺ T-cell response (Pudney et al., 2005, Rowe and Zuo, 2010). More recently lytic CD4⁺ T-cell antigen responses have been found to differ dramatically from their CD8 counterparts with responses widely distributed across IE, E and L antigen targets. This is as one would expect of a conventionally cross-primed response where antigens released from lytically infected cells are acquired and re-presented to the CD4⁺ T-cell repertoire by antigen-presenting dendritic cells (Long et al., 2011).

1.8 Chronic viral infections in childhood

Children in developing countries are often exposed to high numbers of viral infections at an early age such as HIV, CMV and EBV. There is evidence to suggest that the virus host

balance in children infected with these viruses is altered compared to those who are infected later in development, possibly related to the maturation status of the immune system. Indeed even in cases where viruses do not establish classic latent infections, like hepatitis B, primary infection in childhood leads to a much higher rate of chronic infection as opposed to clearance when compared to primary infection in adolescents and adults (Broderick and Jonas, 2003).

A clear example of primary infection with a chronic virus establishing an altered virus host balance in children when compared to adults comes from studying HIV infection. HIV infection in childhood is known to behave differently to primary adult infection. In adults viral loads initially peak and subsequently decline to a set point, which in some cases can be undetectable. They tend to remain around this set-point until such time as the virus host balance shifts and progression to acquired immunodeficiency syndrome (AIDS) occurs (Pantaleo and Fauci, 1995). By contrast childhood HIV viral loads tend to be much higher than adults and the decline to a lower viral load set-point does not occur (Biggar et al., 2001). Slyker *et al.* recently showed that HIV-specific CD8⁺ T-cells activated during early infant infections fail to undergo a transition to a resting phenotype as observed in adult infections, but remain activated in a state potentially vulnerable to apoptosis. Furthermore the presence of activated and apoptosis vulnerable cells in these HIV infected children were associated with CMV co-infection (Slyker et al., 2009).

Perhaps one of the most dramatic impacts on the infant immune system relates to early CMV infection in The Gambia, recently described by Miles *et al.* Here a profound effect on the total CD8⁺ T-cell compartment was demonstrated with an increase in the proportion and absolute number of terminally differentiated total CD8⁺ T-cells, which is maintained for a number of

years (Miles et al., 2008b, Miles et al., 2007, Miles et al., 2008c). The CD4+ T-cell compartment was also affected but less dramatically with only a small increase in cells expressing a highly differentiated phenotype (Miles et al., 2008a). Chronic infection of the elderly with CMV is associated with large expansions in the CD28–CD57+ subpopulation of CD8+ T-cells. This expansion of differentiated and potentially immunosenescent CD8+ T-cells may contribute to an ‘immune risk phenotype’, that is associated with poor responses to the influenza vaccine and to EBV (Brunner et al., 2011, Moss, 2010, Khan et al., 2004). This subpopulation of CD8+ T-cells, CD28–CD57+, was also expanded in CMV infected Gambian infants studied by Miles *et al.* However, they were still able to mount a CD8+ T-cell response to measles vaccine equivalent to those of uninfected infants (Miles et al., 2008b).

In terms of other chronic herpesvirus infections, which are known to induce large antigen-specific responses, Njie *et al.* showed that healthy control children and adults in The Gambia have significantly higher EBV viral loads in PBMCs than healthy UK adults (Njie et al., 2009). In healthy control Ghanaian children EBV-DNA has been detected in plasma, thought to be an indicator of active EBV replication, whereas no EBV-DNA was detected in plasma from control children recruited from Italy (Rasti et al., 2005). It has been readily postulated that acute malaria has an impact on EBV virus host balance, but it appears that regardless of malaria exposure, there is a difference in virus host balance with higher viral load set points seen in equatorial African settings in both children and adults. Recent work in Kenya demonstrated that through multilevel mixed modelling, younger age of EBV infection was a significant predictor for having a higher EBV load in childhood (Piriou et al., 2012).

The dynamics of the immune response to EBV infection when it occurs in childhood are not known. There is a paucity of data in the literature describing both primary and persistent EBV infection in infancy and its implications, which is not surprising given that infections in children are often asymptomatic or do not present with the typical AIM syndrome. The reasons for the age-specific variation in illness severity remain elusive. EBV infection in childhood in Africa has been shown to occur without a significant lymphocytosis and with reduced IgM anti-VCA antibody titres, further compounding the challenge of identifying these children (Biggar *et al.*, 1978a, Biggar *et al.*, 1978b). Fleisher *et al.* screened American children in an out patient setting to compare EBV serology and clinical presentations to that previously detected by Biggar *et al* in Ghana. The clinical symptoms detected in children with serological evidence of current EBV infection determined by the detection of IgM anti-VCA did not differ to those found in children that did not have serological evidence of current EBV infection (IgM anti-VCA negative)(Fleisher *et al.*, 1979). A more recent study conducted by a group at the University of Massachusetts looked at responses in children, aged 20-35 months; indirectly assessing cytotoxic T lymphocyte (CTL) responses using recombinant vaccinia constructs expressing single latent genes (Tamaki *et al.*, 1995). These children were serologically screened and referred to as having had prior exposure to EBV however it is not clear whether they detected antibodies to IgM or IgG anti-VCA nor how they determined their classification of EBV status. Nevertheless, with the caveat of a small sample size, they demonstrated that all children had CTL responses against one or more of the EBNA proteins 3A, 3B and 3C, similar to the latent responses reported for adults. However, a comprehensive description of lytic and latent antigen responses, in the EBV-specific and total T- and B- cell compartments has not been conducted during primary or persistent EBV infections in childhood.

1.9 Epstein-Barr virus and Vaccination responses in childhood

Since their genesis 200 years ago, when Edward Jenner demonstrated that the relatively innocuous cowpox could protect against small pox, vaccines have saved countless lives. There has been a marked increase in the proportion of the world's children who receive their basic vaccines from 15% in 1974 at the start of the Expanded Programme for Immunisation (EPI), to a current 90%, although substantial regional, national and local differences remain (Greenwood et al., 2011). Vaccination has led to the elimination of poliomyelitis and measles from large parts of the world, and complete eradication of small pox. Despite improved availability, some childhood vaccines such as measles have been noted to be less effective in sub-Saharan Africa than in higher income settings (Vitek et al., 1999, Aaby et al., 1990).

EBV is harboured within the memory B-cell pool and it is therefore plausible that it could disrupt the homeostatic maintenance of this population or affect antigen-specific antibody secretion by memory B-cells, and there is some evidence to suggest this. A recent study at the Sukuta field site in The Gambia showed that EBV-infected children had lower antibody responses to measles haemagglutinin and to meningococcus polysaccharide antigens compared to those that were uninfected (Holder et al., 2010). Despite the significant impact of CMV infection in infancy on the phenotype of the total CD8⁺ T-cell population as described above (Miles et al., 2007), CMV did not have any effect on response to vaccination (Holder et al., 2010, Miles et al., 2008b). As mentioned above, in sub-Saharan Africa certain childhood vaccines such as measles are less effective than in high-income settings, suggesting that exploration of the impact of EBV infection in infancy is highly relevant (Aaby et al., 1990, Vitek et al., 1999).

1.10 Burkitt Lymphoma, Lymphoproliferative disorders and other EBV associated Malignancies

What is now known as eBL was discovered by Denis Burkitt in the 1950s during his original observations of a tumour, then thought to be a ‘sarcoma of the jaw’, occurring at a remarkable frequency in East African children (Burkitt, 1958, Burkitt, 1962). Prompted by the unusual restriction of eBL incidence to areas of Africa where arthropod-borne infections such as yellow fever and *P. falciparum* malaria were common, Epstein together with his PhD student, Yvonne Barr, and pathologist Bert Achong began examining tumour biopsies for evidence of infectious agents. While biopsy cells gave uniformly negative results, once permanent BL cell lines were established in culture, a small fraction of the cells were found to contain herpesvirus-like particles. This proved to be a new human herpesvirus, subsequently called EBV (Epstein et al., 1964, Epstein and Barr, 1964). A histologically and clinically very similar tumour was subsequently found in Papua New Guinea, another area holoendemic for *P. falciparum* malaria, and again this tumour proved to be EBV-positive (Moss et al., 1983). Subsequently a sporadic form of BL (sBL) has been observed worldwide as a rare childhood tumour, while in the 1980s an AIDS-associated form of the tumour (AIDS-BL) appeared at a remarkably high incidence in adult HIV-positive patients. All three epidemiologically distinct forms of BL contain the c-myc (proto-oncogene) translocation, but vary in EBV association. Thus, while 100% of the endemic tumours examined have been EBV genome-positive, the EBV association rates for sBL range from 15-20% in Western Europe, where incidence is around 100-fold lower than the endemic tumour, to 85% in some areas of North Africa and Brazil where other parasitic infections are common and where tumour incidence is intermediate between the above two extremes (Gutierrez et al., 1992, Kelly and Rickinson, 2007). Somewhat distinct from this trend is AIDS-BL, which has a very high incidence

among HIV-infected individuals in the West, but where only 30-40% of tumours are EBV genome-positive. Crucially, the situation with respect to EBV and AIDS-BL in equatorial Africa is not yet resolved, but the few cases described so far have all been EBV-positive (Carpenter et al., 2008).

NPC is another geographically restricted EBV associated tumour, occurring in high frequencies in Southern China, Northern Africa and Alaska. The incidence remains high among Chinese people who have emigrated to Southeast Asia or North America, but is lower in Chinese people born in North America compared to those born in Southern China (Buell, 1974, Dickson and Flores, 1985). NPC is a squamous cell carcinoma occurring in the epithelial lining of the nasopharynx first reported in a group of patients in 1901 (Jackson, 1901). Genetic associations with some Class I HLA alleles have been demonstrated in the Chinese population (2000, Lu et al., 2003, Lu et al., 2005, Lu et al., 1990, Ooi et al., 1997). The pathogenesis of NPC is multifactorial, combining genetic and environmental factors.

EBV has been linked to a variety of malignancies over the years including eBL, NPC and Hodgkin lymphoma (Thorley-Lawson and Gross, 2004). EBV utilises multiple oncogenic mechanisms, some of which have been described such as activation of B-cell growth programs, immune evasion and inactivation of tumour suppressors (Young and Rickinson, 2004). Transplant patients illustrate the importance of immune control of EBV reactivation in preventing transformation *in vivo* (Munz and Moormann, 2008). Immunosuppression can cause the immune system to lose control of EBV, which can result in PTLN with uncontrolled B-cell proliferation; whilst restoration of the immune response by relieving immunosuppression can provide a successful treatment (Gulley and Tang, 2010, Loren et al.,

2003, van Esser et al., 2001). EBV is also known to cause lymphomas in those infected with HIV in addition to the aforementioned AIDS-BL. Additionally, an immunoblastic lymphoma, (post-transplant like, frequently involving the CNS) 100% EBV driven appears in the late stage of AIDS (Cote et al., 1997, Levine, 1993), that can be controlled upon initiation of anti-retroviral therapy and restoration of immune function.

EBV has different types of latent gene expression as previously mentioned and distinct forms have been found to manifest in differing tumours (Table 1.1). NPC tumour cells have been shown to express EBNA-1 together with variable levels of LMP proteins, typically LMP-2 positive with some also LMP-1 positive (Fahraeus et al., 1988, Raab-Traub et al., 1983, Young et al., 1988). BL tumours and derived BL cell lines mainly display a Latency I form of infection with expression of a single viral protein, EBNA-1 (Gregory et al., 1990, Rowe et al., 1987), possibly with LMP-1 and EBNA-2 sometimes detectable in a small number of cells; (Niedobitek et al., 1995); interestingly, a minority of BL tumours carry an EBNA-2 deleted virus genome and express EBNA-3A, 3B, 3C in addition to EBNA-1, though the cells retain a classical BL-like phenotype (Kelly et al., 2002, Kelly et al., 2005). In early onset PTLN in immunocompromised transplant patients, EBV generally expresses the full set of latency genes, latency III (Young et al., 1989), and thus appears to represent the *in vivo* counterpart of *in vitro* immortalised lymphoblastoid cells lines (LCLs). However, in later-onset tumours other forms of latency have occasionally been observed, and some EBV-negative forms of PTLN have been described (Dotti et al., 2000, Nelson et al., 2000). In the case of EBV positive Hodgkin disease, Reed-Sternberg cells express high levels of LMP-1 and LMP-2 with EBNA-1 (Niedobitek et al., 1997) in the absence of EBNA-2 expression, which is a Latency II type pattern (Deacon et al., 1993, Murray et al., 1992, Pallesen et al., 1991).

In summary, persistent EBV infection is associated with several different malignancies, BL, NPC and a subset of lymphomas in addition to PTLD. The former, eBL, is found to be endemic in the malaria belt in Africa, peaking in children five to seven years of age. The likely contributing factors to the aetiology of eBL is early primary EBV infection alongside high levels of malaria exposure (Sumba et al., 2010) (Biggar et al., 1978a, Njie et al., 2009, Piriou et al., 2012); both of which are known to cause B-cell hyperplasia.

1.11 *P. falciparum* Malaria: clinical disease and immune pathology

In contrast to EBV's widespread prevalence, malarial infection is clustered in areas determined by climate hospitability for the parasite vector, the *anopheles* mosquito. There are several species of *plasmodium* parasite that infect humans; *ovale*, *malariae*, *vivax*, *knowlesi* and *falciparum*. The latter is responsible for most of the disease burden and deaths, with regions of high endemicity seen throughout sub-Saharan Africa. Malaria is estimated to cause between 300-500 million episodes of acute illness (Amador and Patarroyo, 1996) and more recently the annual mortality for children under five years of age in Africa was estimated at 800 000 deaths (Rowe et al., 2006). Accurate baseline epidemiological data on malaria prevalence is difficult to obtain but remains a pressing issue in order to enable assessment of current control programs (Breman and Holloway, 2007). A recent retrospective analysis of original records in The Gambia demonstrated a progressive reduction in proportion of ward admissions and deaths attributed to malaria, in addition to declining positivity rates reported from out-patient clinics (Ceesay et al., 2008).

While the association between eBL and holoendemic malaria incidence stems back decades (Burkitt, 1969), the relative contributions of EBV infection and malaria exposure to the pathogenesis of eBL still remain to be elucidated. It has been postulated that malarial infections have immunosuppressive consequences; indeed the incidence of salmonella septicaemia is closely related to changes in malaria transmission (Mabey et al., 1987). Furthermore, Greenwood *et al.* demonstrated impaired vaccination responses to tetanus, typhoid and meningococcal vaccines during clinical and asymptomatic malaria infections (Greenwood et al., 1972, Williamson and Greenwood, 1978). Bed net usage decreases all cause mortality in children further suggesting the occurrence of non-specific effects of malaria infection (Phillips-Howard et al., 2003).

The inference that malaria could cause depressed immunity to other infectious pathogens is consistent with early reports that EBV-specific immune responses are decreased during acute malaria infections (Whittle et al., 1984, Moss et al., 1983). A study in New Guinea comparing healthy EBV carriers resident in holoendemic malarial areas to non-malarial areas first suggested that malaria might cause a relative impairment of EBV-specific T-cell immunity. This was seen using an *in vitro* assay that measures the ability of T cells to cause regression of outgrowth of autologous EBV-infected B-cells (Moss et al., 1983). More importantly, Whittle *et al.* working in The Gambia found that PBMCs from patients with acute malaria were completely unable to control outgrowth in regression assays (Whittle et al., 1990, Whittle et al., 1984). This provided a plausible link between malaria and EBV in the pathogenesis of eBL. It has been shown that latent epitope-specific CD8⁺ T-cells are the main effectors responsible for the killing of EBV-infected B-cells in this experimental system (Gudgeon et al., 2005). However, there has been no direct work with modern methods to ask whether

EBV-specific CD8⁺ T-cells are depleted during episodes of acute malaria, or are present but rendered non-functional, or if the lack of regression reflects breakdown at some other point in the complex interactions between infected B-cells, CD4⁺ T-cells and CD8⁺ T-cells that precede the final effector phase in regressing cultures.

The findings of Whittle *et al.* have been indirectly corroborated by further studies. Elevation in EBV VCA antibody titres was found to precede the onset of eBL, (de-The *et al.*, 1978, zur Hausen *et al.*, 1970). The addition of *P. falciparum* extract to PBMCs has been shown to increase EBV B-cell transformation rates *in vitro* (Kataaha *et al.*, 1984, Whittle *et al.*, 1990), and recently data from a smaller study suggests that the cysteine-rich inter-domain region 1 alpha (CIDR1 α) of the *P. falciparum* membrane protein 1 could act as an antigen that can directly induce EBV reactivation, stimulating transition of cells from the latent to the lytic cycle of EBV infection (Chene *et al.*, 2007).

A further study of children in Kenya with differential malaria exposure showed that a smaller proportion of children, five to nine years of age, had EBV-specific IFN- γ responses when living in malaria-holoendemic areas compared to those living in areas where they are only sporadically exposed to malaria (Moormann *et al.*, 2007). More recently diminishing T-cell immunity over time to EBV lytic but not latent antigens in Kenyan children with recurrent *P. falciparum* exposure has been demonstrated (Snider *et al.*, 2012). Furthermore, in the same differentially malaria exposed Kenyan cohorts, no differences in EBV-specific CD8⁺ T-cell frequencies were seen, but children living in regions with holoendemic malaria exposure had more differentiated EBV-specific CD8⁺ T-cell populations than those in sporadically exposed areas (Chattopadhyay *et al.*, 2013). Importantly this phenotypic variation was not detected in

CMV-specific CD8⁺ T-cells nor in the total CD8⁺ T-cell population. Deficiencies in EBNA-1 specific IFN- γ responses, the solitary viral antigen expressed in the majority of eBL tumours, have been detected in children with eBL residing in Kenya compared to healthy children residing in areas of holoendemic and sporadic malaria exposure (Moormann et al., 2009).

Two main hypotheses have been proposed in order to explain how holoendemic malaria impacts on eBL pathogenesis. The first envisages malaria acting as a stimulus for B-cell proliferation, causing expansion of the latently infected B-cell pool, thus increasing both the number and the proliferative status of EBV-carrying B-cells and increasing their chances of c-myc gene translocation and malignant change. The second envisages malaria suppressing EBV-specific T-cell surveillance, again leading to over-expansion of the EBV-transformed B-cell pool and increasing the chances of secondary events leading to lymphoma. Currently there is little direct evidence supporting either hypothesis, and of course it should be borne in mind that both processes could be acting synergistically (Rochford et al., 2005).

Whittle *et al.* has previously suggested that numbers of EBV carrying B-cells were five times higher during acute malaria infections than on recovery and in fact equivalent to numbers seen during AIM (Lam et al., 1991). As noted above, it has also been suggested that the addition of *P. falciparum* extract to PBMCs increased EBV B-cell transformation rates (Kataaha et al., 1984). More recent studies utilising molecular methods have enumerated circulating EBV viral DNA loads in children living in areas holoendemic for malaria exposure and those with acute malaria infections (Donati et al., 2006, Moormann et al., 2005, Rasti et al., 2005, Yone et al., 2006). This heterogeneous group of studies has been conducted in varying sites, Uganda, Kenya, Gabon, The Gambia and Ghana, with EBV viral load

measurements made on various bodily fluids including plasma, whole blood and saliva. In Kenya, the Moorman group identified that children aged one to four years old, living in holoendemic malarious areas had higher EBV viral loads than those resident in an area where they are only sporadically exposed (Moormann et al., 2005). Yone *et al.* analysed whole blood and plasma viral loads in children with acute mild and severe malaria. They found a significant increase in EBV whole blood viral loads during acute mild malaria infection. This declined on convalescence and was lower still at six months post infection. A similar trend was seen with severe malaria but this did not reach significance. They found few children with detectable plasma viral loads but it was not clear if these were intact virions or fragments of DNA (Yone et al., 2006).

More recently in The Gambia, Njie *et al.* looked at PBMC viral loads during acute malaria infection, but found no significant difference in acute versus convalescent viral loads. However, they did show a five-fold increase in EBV viral loads compared to the healthy control group of Gambian children (Njie et al., 2009). Rasti *et al.* and Donati *et al.* both assessed plasma EBV viral loads with the latter also looking at salivary loads. They both showed significant trends of decreasing plasma EBV viral loads on recovery. However, Rasti *et al.* did not use age and sex-matched controls and Donati demonstrated that the material they were measuring was naked DNA and not in intact virions (Donati et al., 2006). The source then of the viral DNA detected in the above studies is not yet clear. Whether it reflects an increase in the numbers of circulating latently infected B cells, or the presence of lytic EBV replication in circulating cells, or the detection of EBV DNA fragments derived from latently-infected cell death within tissues as is seen in patients with EBV-positive tumours, is not

known (Berger et al., 2001). The mechanism(s) behind the source of this apparent elevation in viral loads therefore need to be further characterised.

1.12 Summary

In relation to the pathogenesis of eBL, exploring the dynamics of the immune response to EBV during acute *P. falciparum* infection is of significant importance. It is of great interest then to determine how cellular immune responses to EBV behave in malaria exposed African children in terms of number, function and phenotype, using contemporary analytical methods to inform our understanding of the mechanisms controlling the pathogenesis of eBL.

Furthermore, how primary EBV infection develops and how the cellular immune response controls it in African children is unknown. Primary asymptomatic EBV infection in childhood is known to differ from that seen in adolescence in that AIM is rarely seen in children upon primary EBV infection. Exploration of how cellular immune responses differ in childhood primary infections may shed light on the mechanisms behind the age-specific clinical presentation of primary EBV infection. An understanding of the immunodominant immune responses in childhood would aid vaccine development for this oncogenic virus, or allow the development of interventions to alter the virus host balance in these children potentially at risk of eBL. Similarly, whether early childhood EBV infection, which is known to encode proteins that can manipulate the immune system, affects the function of the immune system, in terms of responses to vaccination is of importance in view of its high incidence and prevalence at an age when African children are receiving their primary vaccination schedules.

The data presented in this thesis were gathered firstly to further explore these questions. The first phase of the study examines EBV-specific CD8⁺ T-cell responses in Gambian children during acute malaria infection and at convalescence. T-cell function was studied by regression assay analysis and also more contemporary immune measures, namely tetramer staining and ELISPOT analysis. These assess numbers and functional competence of EBV-specific CD8⁺ T-cells in children respectively.

In the second phase of this study, the dynamics of primary EBV infection in childhood is explored. This represents a detailed study of EBV-specific T-cell responses in a cohort of children followed longitudinally and describes cellular immune responses in Gambian children having undergone recent primary EBV infections. It also fortuitously captured several children undergoing asymptomatic ‘silent’ seroconversion. This provides a comprehensive analysis of EBV-specific T-cells in these children, including a characterisation of the immunodominant responses to a broad range of lytic and latent EBV antigens, viral genome loads and detailed *ex-vivo* phenotypic analysis of both total and EBV-specific CD8⁺ T-cells.

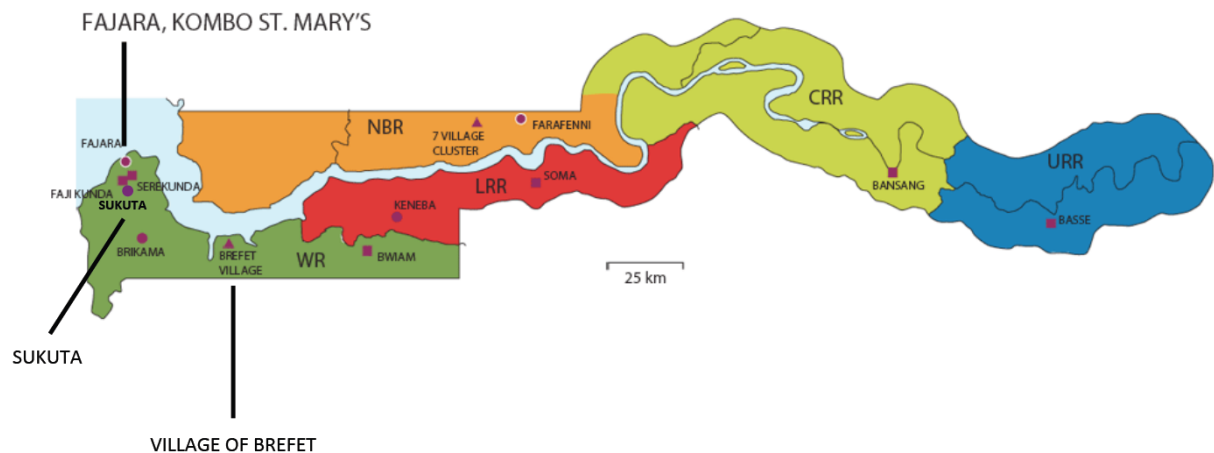
Lastly, the potential impact of EBV infection in childhood, an infection that primarily infects and persists in B cells, on B-cell phenotypes and the response to a vaccine challenge with a pentavalent (Diphtheria, Tetanus, *Bordetella pertussis*, Hepatitis B and *Haemophilus influenzae b* (DTP, HepB and Hib)) booster vaccination given at 14-18 months is explored. This determined whether EBV infection in early life has any impact on the function of the humoral response to vaccine challenge.

CHAPTER 2 : METHODS

The body of work contained in this thesis is broadly divided into two main sections, Phase I (Chapter 3) and Phase II (Chapters 4 and 5). Although techniques used were similar in both phases of study there were some significant differences in the methodologies. These have been detailed under the appropriate section headings and as indicated in the text Phase I and II are dealt with separately where necessary.

2.1 Study cohorts

The study participants were from two peri-urban and one rural community based in The Gambia within West Africa, locations illustrated in Figure 2.1.



Western Region (WR), North Bank Region (NBR), Lower River Region (LRR), Central River Region (CRR), Upper River Region (URR).

Figure 2-1 Map of The Gambia showing the location of the study sites.

Shown are the sites for Phase I, the MRC Laboratories Fajara and the Village of Brefet and for Phase II Sukuta.

Thirty-two Gambian children (aged 1–15 years) enrolled into studies of severe and uncomplicated malaria in the outpatient department of the Medical Research Council (MRC) in Fajara, a peri-urban area, were tested in the first phase of this study (Walther et al., 2009). All presented with uncomplicated *P. falciparum* malaria, defined as a temperature $>37.5^{\circ}\text{C}$ within the last 48 hours, and ≥ 5000 parasites/ μl detected by slide microscopy in the absence of any signs defining severe malaria as defined by WHO (2000). Thirty-two age- and sex-matched healthy aparasitaemic controls were recruited from Brefet, a rural community where malaria transmission has dropped considerably over the last three years (Ceesay et al., 2008, Ceesay et al., 2010, Satoguina et al., 2009). Brefet is approximately 55km from Fajara, where the MRC laboratories are housed. All patients received standard care according to the Gambian Government Treatment Guidelines.

Phase II of the study was conducted in a peri-urban MRC clinic, Sukuta, situated within the Government Sukuta Health Centre, serving a low income population living in crowded conditions. This is a distance of 10 km from Fajara. A cohort of 120 children aged between 14 and 18mths were recruited when they attended the local government health Centre for their routine booster vaccination of DTP. All children were weighed, measured, had baseline observations (temperature, heart rate, weight and length) and a clinical examination performed. Any child found to be unwell or have a weight below that specified on the local Infant Welfare Card Growth Chart was not recruited into the study. Treatment was provided where necessary by MRC nursing staff and the study clinician. Children were brought back one week later to receive the Easy Five Panacea Biotec vaccine (Pentavalent vaccination (DTP, HepB, Hib)). They were subsequently invited to return a week after vaccination and

again at six months to undergo further blood sampling. All blood samples were batched and returned to the MRC laboratory in Fajara within six hours of being drawn.

2.2 Transport, PBMC isolation and cryopreservation

For phase I *P. falciparum* infected children were bled on admission (D0) and after 28 days (\pm three days); controls were bled at one time point only. A 500 μ l volume of blood was collected into an EDTA microtainer® (BD) for a full blood count (FBC) and DNA extraction. A further 4mls was collected into heparinised vacutainers® (BD). These were then batched and transported back from Brefet or deposited directly in the laboratory if taken in Fajara. A 'thick and thin blood smear' was prepared from the heparinised whole blood sample for all study participants to determine the species and magnitude of the parasitaemia. The whole blood was then centrifuged for 5 mins at 250 \times g at room temperature with low brake. The plasma layer was collected and cryopreserved in 2ml aliquots for EBV VCA and 19-Kilodalton Merozoite Surface Protein 1 (MSP1-19) malarial antigen enzyme linked immunosorbent assays (ELISAs) to be performed (Section 2.8.1 and 2.8.2). The remaining blood was diluted 1:1 with RPMI and then layered onto 4ml of Nycoprep™ in 15ml falcon tubes. This was centrifuged at 800 \times g for 30 mins with no brake. The lymphocyte interphase was harvested and washed twice in RPMI by centrifuging at 250 \times g for 7 mins. These PBMCs were then used to set-up IFN- γ ELISPOT assays to pools of lytic and latent EBV peptides (Table 2.3) and for tetramer and surface flow cytometric staining (Section 2.16).

During phase II 5mls of blood was collected from each child into vacutainers® containing EDTA (BD). A 500 μ l aliquot was removed and used to obtain a FBC on each child using a M-series M16/M20 Haematology Analyser (Medonic, Sweden). A further 250 μ l aliquot was

removed and whole blood flow cytometric staining performed (Section 2.13). The remaining blood was layered on to 4mls of LymphoprepTM (Axis-Shield, UK) in 15ml Leucosep[®] tubes (Greiner Bio-One, UK). These were centrifuged for 15 min at 800 x g with the brake off. The plasma layer was removed, and cryopreserved in 2ml aliquots and stored at -70°C for downstream serology (Section 2.8.1 and 2.8.3). The lymphocyte interphase was harvested and washed twice in RPMI by centrifuging at 250xg for 5 mins. Cells were counted and re-suspended in freezing medium (FCS (Sigma-Aldrich) supplemented with 10% (v/v) dimethyl sulfoxide (DMSO)) at approximately 5×10^6 /ml. Graded PBMC cryopreservation was undertaken utilising a 'Mr Frosty' freezing chamber (Thermo Fisher Scientific, UK) containing 100% Isopropan-2-ol. Cryovials containing PBMCs were placed in the freezing chamber and this placed in the -70°C freezer overnight, prior to transferring to liquid nitrogen (LN2) the following day. Cryopreserved PBMCs in LN2 and plasma (on dry ice) were transported to the Weatherall Institute for Molecular Medicine (WIMM) and the Institute for Cancer Studies in Birmingham for multi-colour flow cytometry staining (Sections 2.14 and 2.16) and estimation of EBV viral load (Section 2.6) respectively.

2.3 Maintenance of B95.8 cell line and harvesting virus

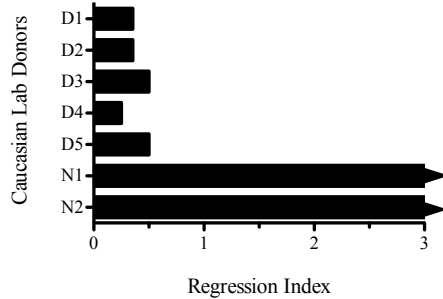
The B95.8 cell line, a cell line initiated by exposing marmoset blood leukocytes to EBV extracted from human leukocyte lines was cultured providing a continuous source of high titre transforming EBV for regression assays. Cryopreserved cells free of mycoplasma obtained from the Institute for Cancer Studies in Birmingham were thawed rapidly into 10mls of warm RPMI supplemented with 10% (v/v) heat-inactivated FCS, 2mM L-glutamine (Sigma), 1% Pen Strep (100U/ml penicillin and 0.1mg/ml streptomycin final concentration) (Sigma) (R10) and washed once in R10. The cells were re-suspended at $1.0\text{-}2.0 \times 10^6$ cells/ ml depending on

cell numbers recovered. They were sub-cultured twice a week by splitting cells 1:4 to maintain a density of approximately 1×10^6 cells/ ml. Some subcultures were not split for a period of one week and virus from their filtered supernatant was used to perform regression assays in real-time. The presumption was made that they contained saturating amounts of EBV.

2.4 Regression Assays (Phase I)

Regression assays were carried out on stored cryopreserved PBMCs from the 2008 malaria season and fresh cells *ex vivo* from healthy controls in parallel. Cryopreserved PBMCs from uncomplicated and severe *P. falciparum* malaria cases recruited from the same study platform (Walther et al., 2009) were used. Regression assays were performed by infecting PBMCs with B95.8 strain EBV preparations (Section 2.3) for one hour at 37°C, agitating every 15-20 mins and seeding six replicates into U-bottom micro test plates in doubling dilutions from 4×10^4 to 1.2×10^3 cells/well in R10. In two replicate wells cyclosporine A (CSA) was added to a final concentration of 0.1µg/ml. Parallel cultures of uninfected PBMCs were set up as controls. A half change of medium was performed weekly on all cultures and they were observed over a period of 4-5weeks. The strength of regression was expressed as the initial cell seeding necessary to give a 50% incidence of regression among replicate wells as calculated using the Reed-Muench formula (Reed, 1938). Previous methods (Gudgeon et al., 2005) described used 10-fold greater cell numbers in flat bottom micro test plates, however for this study the assay was miniaturised with no significant difference in the magnitude of regression detected (Figure 2.2).

A. Flat Bottom



B. U-Bottom Wells

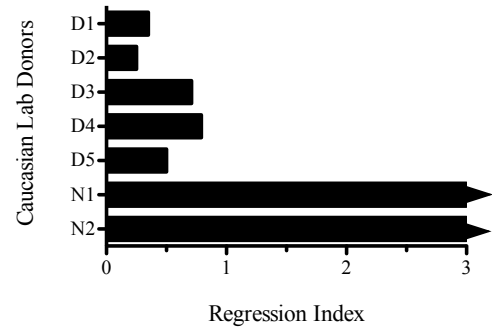


Figure 2-2 Representative samples illustrating Standard (A) and miniaturised (B) Regression assays performed simultaneously on seropositive (D1-D5) and seronegative (N1-2) Caucasian Donors (n=5). Wilcoxon rank test, $p=0.50$.

2.5 Extraction of DNA (human and viral)

DNA was extracted from 200µl of whole blood and 1×10^6 PBMCs for phase I and phase II respectively using the QIAmp DNA Blood Mini kit (Qiagen, UK). A 20µl volume of proteinase K was pipetted into the bottom of a 15ml micro centrifuge tube and 200µl of sample (whole blood or 1×10^6 PBMCs in 200µl of phosphate buffered saline (PBS)) and 4µl RNase A stock solution (100mg/ml) were added. Following this 200µl buffer AL was added and mixed by pulse-vortexing for 15 secs. Samples were incubated at 56°C for 10 mins. A 200µl volume of ethanol (96-100%) was added to the sample and mixed again by pulse-vortexing for 15 seconds. The contents were then applied to the QIAamp mini spin column (in a 2ml collection tube) without wetting the rim and centrifuged at 6000xg (8000rpm) for 1 min. The columns were then placed in a clean 2ml collection tube and the filtrate tube discarded. Each column was opened and 500µl of Buffer AW1 was added again without wetting the rim. The cap was closed and columns were centrifuged at 6000xg (8000rpm) for 1min. The columns were placed in clean 2ml collection tubes and the filtrate discarded. Each

column was opened and 500 μ l of Buffer AW2 was added without wetting the rim. Caps were closed and the columns were centrifuged at full speed (20,000xg; 14,000 rpm) for 3 mins. Columns were placed in clean collection tubes and centrifuged at full speed for a further 1min. Columns were then placed in DNase and RNase free 1.5ml micro centrifuge tube and the collection tubes containing the filtrate discarded. Each column was opened and 200 μ l of buffer AE was added. Columns were then incubated for 5 mins at room temperature and centrifuged at 6000xg (8000rpm) for 1min. The quantity (3-6 μ g per million cells) and quality (260/280 ratio >1.6-1.8) of DNA extracted from each sample was estimated by measurement on a Nanodrop spectrophotometer (Thermo Scientific, Nanodrop products, 3411 Silverside Rd, USA) and then stored at -20°C and shipped on dry ice to the Institute for Cancer Studies, Birmingham to determine the EBV viral loads (Section 2.6).

2.6 EBV real time Quantitative PCR

EBV genome loads were assayed by quantitative real-time PCR as previously described (Junying et al., 2003). Target DNA sequences were amplified and monitored in real-time by changes in fluorescence intensity of dual-labelled Taqman probes. Primers amplifying a fragment of the EBV DNA polymerase (Pol) gene in combination with a FAM-labelled probe detected EBV DNA. Human beta-2-microglobulin (β 2m) gene in combination with a VIC-labelled probe was amplified to determine the input of cellular DNA in each sample and normalise for variations in cell number input into the assay. Primer and probe sequences were obtained from Applied Biosystems (UK) (Table 2.1). A multiplex PCR reaction was used to detect EBV Pol and control β 2m sequences simultaneously; the β 2m reaction was primer limited to avoid depletion of PCR reagents. Reactions were conducted in triplicate in 25 μ l volumes containing 12.5 μ l Taqman Universal master mix (Applied Biosystems), 2.5 μ l

(2µM), forward and reverse Pol primers, 1µl (5µM) FAM-labelled Pol probe, 0.5µl (3µM) β2m forward primer, 0.5µl (4µM) β2m reverse primer, 0.5µl (5µM) VIC-labelled β2M probe and 5µl test DNA. The reaction mixtures were amplified for 40 cycles (15 secs at 95°C, 60 secs at 60°C) following the activation of the uracil-*N*-glycosylase (2 min at 50°C) and the Amplitaq Gold (10 min at 95°C). The reactions were run on an ABI Prism 7500 real time PCR system (Applied Biosystems).

Table 2-1 *EBV real-time qPCR primer and probe sequences.*

EBV Polymerase	
Forward primer	5' CTT-TGG-CGC-GGA-TCC-TC 3'
Reverse primer	5' AGT-CCT-TCT-TGG-CTA-GTC-TGT-TGA-C 3'
POL probe	5' (FAM) CAT-CAA-GAA-GCT-GCT-GGC-GGC-CT (TAMRA) 3'
β-2-microglobulin	
Forward primer	5' GGA-ATT-GAT-TTG-GGA-GAG-CAT-C 3'
Reverse primer	5' CAG-GTC-CTG-GCT-CTA-CAA-TTT-ACT-AA 3'
β2M probe	5' (VIC)-AGT-GTG-ACT-GGG-CAG-ATC-ATC-CAG-CTT-C (BHQ) 3'

Data was analysed using the 7500 system v1.4 software. The alterations in FAM and VIC fluorescence intensity (corresponding to the amplification of Pol and β 2M, respectively) were used to determine the corresponding Ct value for each sample. This was defined as the cycle number at which the sample amplification curve crossed a threshold (Pol 0.08 and β 2M 0.04). The Ct value is proportional to the number of copies of DNA fragment starting material and therefore allowed for the number of Pol and β 2M copies in the sample to be deduced from a standard curve. Standards were prepared by serial dilutions (10^4 , 10^3 , 200, 40, 10, 5, 2) of Namalwa BL DNA (diploid Namalwa cells carry two EBV genomes and each cell contains 6.6pg DNA); a calibration curve was plotted with Ct values (y-axis) against \log_{10} of Pol or β 2M copy number (x-axis). This was used to extrapolate Pol and β 2M genomes in the unknown samples from their Ct values. All test samples were analysed in triplicate and each experiment included DNA samples from EBV-positive and EBV-negative donors, in addition to a no template control. Samples were considered negative if the Ct values exceeded 37 in $\geq 2/3$ reactions. Results were expressed as EBV genome copies per 1×10^6 PBMCs correcting for the PBMC percentage using the FBC where whole blood was used for extraction (phase I). This assay reproducibly detects as little as two EBV genomes (Junying et al., 2003).

2.7 *Plasmodium falciparum* parasite PCR

P. falciparum parasite loads were assayed in the Gambia using a previously described quantitative PCR method (Rougemont et al., 2004). The 157- to 165-bp segment of the plasmodial 18S genes was amplified using primers and probes kindly donated by Davis Nwakanma, Malaria Programme, MRC Gambia (Table 2.2).

Table 2-2 *Plasmodium falciparum* parasite PCR primers and probes.

<i>P.falciparum</i> 18S gene	
Forward primer	5' GTTAAGGGAGTGAAGACGA TCAGA 3'
Reverse primer	5' AACCCAAAGACTTTGATTTC TCATAA 3'
Probe	5' (FAM) AGCA ATCTAA AAGTCACCTC GAAAGATGAC T (TAMARA) 3'

Single assays were performed in final volumes of 25µl containing 5µl of DNA, 0.3µM of forward and reverse primers, 0.08µM probe and 12.5µl Qiagen master mix per reaction. Amplification and detection of the amplified product was performed on an ABI Prism 7700 (Applied Biosystems, UK). The following conditions were used for the PCR: an initial step at 95°C for 15 mins and 45 cycles of 94°C for 15secs and 60°C for 1min. If the fluorescence signal did not increase within 40cycles, the sample was considered to be negative.

2.8 Serology

2.8.1 ELISA and Immunofluorescence assays for EBV

For phase I, IgG reactivity to EBV VCA was measured using a commercial ELISA kit (DeMeditech, DEEBV01) performed in the MRC laboratories, Fajara. All plasma samples were diluted 1:101 with sample diluent prior to being assayed in duplicate. A 100µl volume of each kit calibrator (10, 20, 110 and 170 AU/mL) and sample were dispensed into respective wells pre-coated with EBV VCA p18 synthetic peptide and incubated for 1hour at 37°C. Well contents were then discarded and wells washed five times with 300µl wash buffer (PBS Tween[®] 20 and preservatives). A 100µl volume of enzyme tracer (affinity –purified IgG

raised in goats to human IgG conjugated to horseradish peroxidase (HRP), TRIS buffer, BSA, stabilisers and preservatives) was added to all except the blank well and incubated for 1 hour at 37°C. Then 100µl of chromogen/substrate solution (tetramethylbenzidine/hydrogen peroxide) was added to all wells and incubated for 30 mins at room temperature away from direct sunlight. A 200µl volume of stop solution (0.4N sulphuric acid solution) was added into all wells in the same order and at the same rate as the chromogen/substrate solution was added. The absorbance of the specimens was measured with a photometer at 450/630 and 405/630nm within 30 mins of adding the stop solution. A calibration curve was calculated, using kit calibration samples, with absorbance on the y-axis and concentration of IgG to VCA on the x-axis expressed in AU/mL. Cut off for positivity was calculated using the upper limit of the 99% confidence interval obtained from simultaneously screening ten known EBV negative medical student plasma samples kindly donated by Alison Leece, Institute for Cancer Studies, Birmingham.

In the Phase II study immunofluorescent staining was performed to detect EBV IgG VCA antibody's using an in-house assay at the Institute for Cancer Studies in Birmingham. P3HR1 (EBV IgG +ve) and BJab (EBV IgG –ve) cells were cultured, spun down and re-suspended in PBS at approx. 10^7 /ml. 10µl of cell suspension (10^5 cells) was placed in each hole of an SMO11 microscope slide. The slides were air dried in a warm room and fixed in ice-cold acetone for 10 mins. Slides once dry were stored at -20°C. The test plasma was diluted 1/10, 1/20 and 1/40 then 20µl of diluted plasma was placed into the respective holes and slides were incubated in a moist chamber at 37°C for one hour. Slides were then washed in PBS twice for 10 mins at a time, using a Coplin jar and magnetic stirrer. Slides were wiped avoiding cell smear and 10µl of Sigma F0132 anti-human IgG (γ chain specific) affinity isolated

fluorescein isothiocyanate (FITC) antibody added to each hole (1/50) and incubated at 37°C for one hour. Slides were washed twice in PBS for 10 mins at a time, mounted using Dabco, glycerol and PBS and viewed under an *ultraviolet (uv)* microscope. Patients were identified as either positive or negative.

The phase II IgM VCA immunofluorescence assays were kindly performed by Karen McAulay at The LRF Virus Centre, Centre for Virus Research, University of Glasgow using their in-house method (Macswen et al., 2010). Annette Pachnio using an in-house ELISA at the Institute for Cancer Studies, Birmingham, kindly determined CMV antibody status.

2.8.2 ELISA assay for MSP1-19 antibody (Phase I)

Total IgG to MSP1-19 was measured on Phase I control and study plasma samples using an in-house ELISA method at MRC Gambia. Immulon4 plates were coated overnight at 4°C with 100µl of MSP1-19 antigen in coating buffer (15mM Na₂CO₃, 35mM NaHCO₃, pH 9.3) at a concentration of 0.5µg/ml (donated by the MRC Gambia malaria programme). After three washes with wash buffer (PBS with 0.05% Tween20 (Sigma)), the plates were blocked with 200µl blocking buffer (1% skimmed milk (Marvel UK) in wash buffer) for three hours at room temperature. The plates were washed a further three times and plasma added diluted at 1:1000 in blocking solution and incubated overnight at 4°C. Plates were washed six times and incubated for three hours at room temperature with 100µl of anti-human IgG HRP (Dako Ltd, Ely. UK) diluted 1:5000 in blocking solution. The plates were washed six times and 100µl of substrate (0.4mg/ml o-phenylenediamine dihydrochloride (OPD) Sigma); 0.08% H₂O₂ (Sigma) in developing buffer (24.5mM citric acid monohydrate, 52mM Na₂HPO₄, pH 5.0) was added for a further 20 min incubation at room temperature. The reaction was stopped

with 50µl of 2M H₂SO₄ and the optical density (OD) measured at 490nm. All samples were run in duplicate. A pool of sera from 20 malaria-exposed donors was used as a positive control. A seven point standard curve using a five-fold serial dilution of the pool (starting at 1:50) was used to convert OD values into arbitrary units. Antibody levels in sera from 20 non-exposed Europeans were used to define a cut-off (mean OD plus two standard deviations) for a positive response.

2.8.3 ELISA for Hepatitis B antibody (Phase II)

In phase II, Hepatitis B surface antibody (anti-HBs) was measured using commercial ELISA kits (DiaSorin, ETI-AB-AUK-3, P001603) at the MRC laboratories, Fajara. All pre-vaccination (visit one) plasma samples were assayed neat and post-vaccination plasma samples were diluted 1:4 and 1:100 with negative control serum supplied in the kit. A 100µl volume of incubation buffer (PBS, Bovine serum albumin, Triton X-705 and preservatives) was dispensed in all wells except the blank. A 100µl of each calibrator, sample and negative control were dispensed into respective wells pre-coated with heat-treated human Hepatitis B surface antigen (HBsAg) and incubated for two hours at 37°C. Well contents were then discarded and wells were washed five times with 300µl wash buffer (PBS Tween[®] 20 and preservatives). A 100µl volume of enzyme tracer (heat treated human HBsAg conjugated to horseradish peroxidase (HRP), TRIS buffer, BSA, stabilisers and preservatives) was added to all except the blank well and incubated for one hour at 37°C. Then 100µl of chromogen/substrate solution (tetramethylbenzidine/hydrogen peroxide and citrate buffer) was added to all wells and incubated for 30mins at room temperature away from direct sunlight. A 200µl volume of blocking solution (1N sulphuric acid solution) was added into all wells in the same order and at the same rate as the chromogen/substrate solution was added.

The absorbance of the specimens was measured with a photometer at 450/630 and 405/630nm within 30 mins of adding the stop solution. A calibration curve was calculated with absorbance on the y-axis and concentration of anti-HBs on the x-axis expressed in AU/mL. Sample measurements were then multiplied by a dilution factor where appropriate. It is generally accepted that anti-HBs concentration above 10AU/L is indicative of either resolution of a past infection or positive response to vaccination.

2.9 Multiplex immunoassay for simultaneous quantification of serum IgG antibodies to *Bordetella pertussis*, diphtheria and tetanus (Phase II)

Pre- and post-vaccination serum IgG antibody levels against Diphtheria toxin (Dtx) and Tetanus toxin (Ttx), and the *Bordetella pertussis* antigens Pertussis toxin (Ptx), Filamentous Haemagglutinin (FHA), Fimbriae and P69 Pertactin (Prn), were performed with the assistance of Pieter G.M. van Gageldonk at the Laboratory for Infectious Diseases and Perinatal Screening, National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands using their in-house assay as previously described (van Gageldonk et al., 2008). Reagents, reference and control sera, purified Ptx, FHA, Prn, Fimbriae, Dtx and Ttx coupled to activated carboxylated microspheres were kindly donated by the RIVM. Pre- and post vaccination plasma and in-house control sera were diluted 1/200 and 1/4000 in PBS containing (v/v) 0.1% tween-20 and (w/v) 3% BSA. Each dilution of 25µl of standard and plasma sample was mixed with an equal volume of conjugated microspheres (4000/beads/region/well) in a 96-well Multiscreen HTS filter plate (Millipore Corporation, Billerica, MA) and incubated for 45mins at room temperature in the dark at 600rpm on a plate shaker. The beads were collected by filtration through a vacuum manifold and washed three times in a 100µl of PBS. A 50µl volume of a 1/200 dilution of R. Phycoerythrin (R-PE)

conjugated anti-human IgG in PBS was added to each well and incubated while shaking for 30 mins. After two washes beads were re-suspended in 100µl of PBS and shaken before analysis on a Bio-Plex 200, using Bio-Plex manager software version 4.1.1 (Bio-Rad Laboratories, Hercules, CA). For each of the IgG antibody levels the median fluorescence intensity (MFI) was converted into EU/ml or IU/ml using a 5-parameter logistic standard curve (log-log) for each bead region/standard.

Pre- and post-vaccination serum IgG antibody levels against Hib polysaccharide were kindly performed by Gaby P. Smits at the RIVM.

2.10 HLA typing

In Phase I low-resolution class I HLA typing was performed at The MRC Gambia laboratories using sequence-specific primers (SSP) for the HLA-alleles of interest, namely A2, B35, B8 and B53. These were selected based on their frequency in the Gambian population (Figure 2.3), determined by serological typing (Allsopp et al., 1992) and recently confirmed using high resolution sequence typing (Louis-Marie Yindom, unpublished data), and availability of known immunodominant EBV epitopes restricted through these class I HLA alleles (Table 2.3).

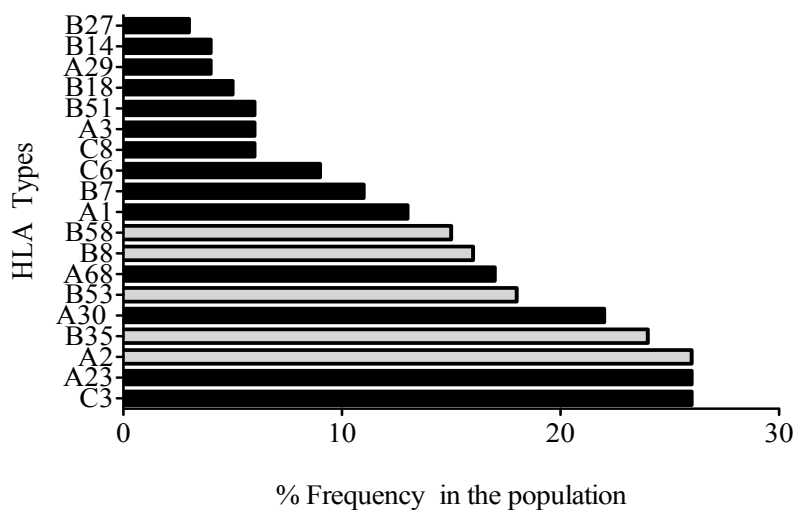


Figure 2-3 Frequencies of HLA-alleles in 900 Gambian Donors.

Grey bars represent HLA restrictions for known EBV-specific CD8+ epitopes used in phase I

Table 2-3 Known CD8+ EBV peptides restricted through common HLA-alleles in The Gambia.

EBV Protein	Peptide Pool	Epitope sequence	Epitope co-ordinates	HLA restriction
EBNA1	Latent	HPVGEADYF	407-415	B53
EBNA1	Latent	HPVGEADYFEY	407-417	B35
EBNA3A	Latent	QAKWRLQTL	158-166	B8
EBNA3A	Latent	YPLHEQHGM	458-466	B35
LMP2	Latent	FLYALALL	356-364	A2
LMP2	Latent	CLGGLTMTV	426-434	A2
BRLF1	Lytic	YVLDHLIVV	109-117	A2
BZLF1	Lytic	EPLPQGQLTAY	54-63	B35
BMLF1	Lytic	GLCTLVAML	280-288	A2

Note. – EBNA, Epstein-Barr Nuclear Antigen. LMP, Latency Membrane Protein.

The reagents and primer mixes for the HLA-alleles of interest and control primers, to a 796 base pair fragment from the third intron of HLA-DRB1, were kindly donated by Dr Louis-Marie Yindom. The PCR method was based on previously described methods for HLA phototyping (Bunce et al., 1995). Relevant primer mixes (5µl) were aliquoted into 1-16 wells of a non-skirted 96-well PCR plate. Each reaction contained 10xbuffer pH8.5 (Tris-HCl, (NH₄)₂SO₄, 1% Tween20®), MgCl (50mM), deoxynucleotide triphosphates (dNTPs) (25mM), 1µg of sample DNA and 0.8µl Bioline Taq. The reaction mix (5µl) was added to the primer mixes giving a final reaction volume of 10µl per well. Plates were then sealed and run on a thermocycler with a heated lid. The cycling parameters were as follows: 1 minute at 96 °C followed by 5cycles of 50 seconds at 96 °C, 50 seconds at 70 °C, 50 seconds at 72 °C, followed by 21 cycles of 50 seconds at 96 °C, 50 seconds at 65 °C, 50 seconds at 72 °C followed by 4 cycles of 50 seconds at 96 °C, 50 seconds at 55 °C and 130 seconds at 72 °C. The final product (8µl) plus loading buffer was then run on a 2% agarose gel (400ml 0.5M TBE, 8g agarose, 8µl ethidium bromide) at 300v for 20-30 mins and viewed under a *uv* light (Figure 2.4). The pattern of wells containing product allowed us to determine if the sample was one of the HLA-types of interest.

In Phase II HLA typing limited to five alleles was performed on donors identified as being IgM VCA positive by serology and found to be responders in the IFN-γ ELISPOT Assay. Annette Pachnio at the Institute for Cancer Studies, Birmingham kindly performed this assay (Figure 2.5).

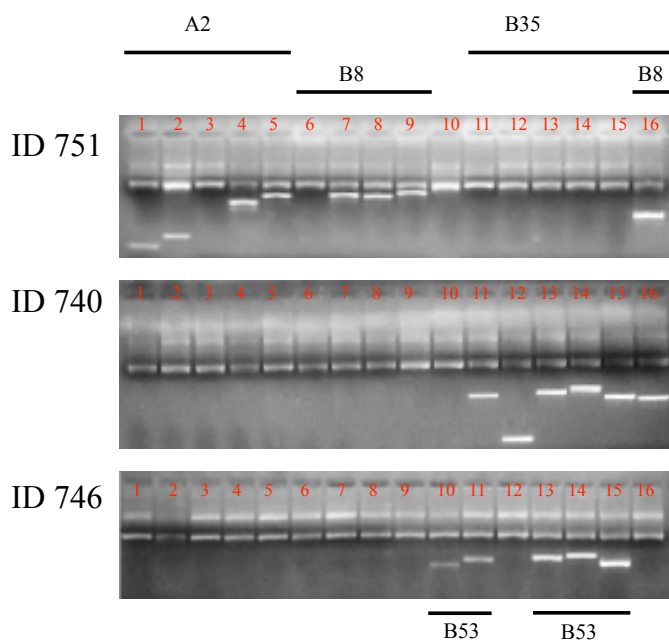
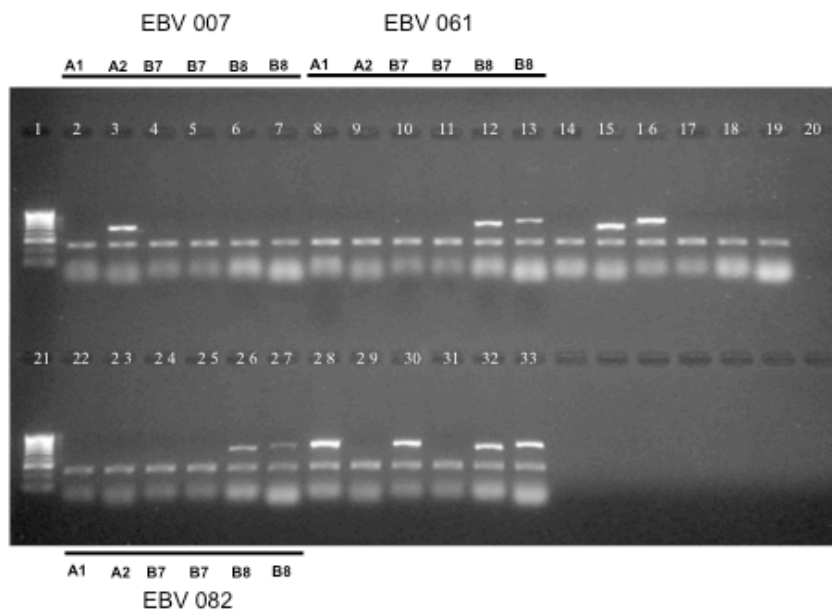


Figure 2-4 Examples of low resolution HLA typing for donors ID751, ID740 and ID746 illustrating HLA types A2 and B8, B35 and B53 respectively (phase I)



Lanes 1+21: DNA Hyperladder IV

Lanes 2-7: EBV -007

Lanes 8-13: EBV 061

Lanes 14-19: HV003 (+ve control: HLA-A2, -B7) – healthy donor control

Lanes 22-27: EBV-082

Lanes 28-33: HV004 (+ve control: HLA-A1, -B7, -B8) – healthy donor control

Well order per sample:

HLA-A1, -A2, -B7, -B7, -B8, -B8

Figure 2-5 HLA mini-typing illustrating that samples ID007, ID061 and ID082 are A2, B8 and B8 respectively (phase II)

2.11 Thawing cells (Phase II)

Cryopreserved PBMCs were thawed rapidly by drop-wise addition of 10mls warmed (37°C) FCS. Following centrifugation at 1600rpm for 5 min, cells were incubated in RPMI (R0) and 60µg/ml DNase solution (Type IV (Sigma)) for 15 mins at 37°C. Cells were washed and re-suspended in R10 and viable cells counted by trypan blue exclusion. Cells for ELISPOT assays were rested for an additional four hours at a concentration of 10^6 /ml, after which cells were washed once in R10. Viable cells were quantified and re-suspended in R10 at 10^6 viable cells /ml. Only samples with cell viabilities $\geq 80\%$ were used in ICS assays. The majority (76%) of samples had cell viabilities of 80–99% after resting for four hours. Cells were immediately used in stimulations for intracellular cytokine staining or tetramer multi-colour flow cytometry assays (Section 2.14 and 2.16). Rested cells were used for the IFN- γ ELISPOT assays.

2.12 Interferon-gamma (IFN- γ) Enzyme-linked ImmunoSpot (ELISPOT) assays

Based on polymorphisms in genes, some of which encode known CD8+ epitopes, two types of EBV can be distinguished. Epitopes used in this study have been defined based on work with type 1 EBV strains in Caucasians. While we did not sequence the Gambian strains of EBV in our donors, Njie *et al.* described previously that 90% of Gambian donors they studied carried type 1 EBV (Njie *et al.*, 2009) indicating that the use of peptides derived from type 1 EBV is a reasonable choice. In addition there is extensive homology between the two subtypes and cross-reactive responses have been recognised (Bornkamm *et al.*, 1980, Sample *et al.*, 1990, Zimmer *et al.*, 1986, Brooks *et al.*, 2000).

In Phase I, IFN- γ producing EBV-specific lymphocytes were enumerated using the ELISPOT technique at MRC Laboratories, The Gambia. PBMCs were cultured in growth medium (RPMI, containing 10% human AB serum). 96-well, flat-bottomed (Millipore, Bedford, Mass, USA) plates were pre-coated with anti-IFN- γ mAb 1-DIK (MABTECH, Stockholm, Sweden) at 37°C for four hours. Plates were then washed six times with RPMI and blocked with R10 for 30 mins at 37°C. *Ex vivo* responses against lytic and latent pools of EBV peptides (Table 2.3, obtained from the WIMM, Oxford), and Phytohaemagglutinin (PHA), both at a final concentration of 5 μ g/ml, were screened at 10⁵ PBMCs per well in duplicate. An equivalent concentration of DMSO was used as the negative control. Plates were incubated overnight at 37°C, 5% CO₂. The cells were discarded the following day, and a biotinylated anti-IFN- γ mAb, 7-B6-1 biotin (MABTECH), was added at 1 μ g/ml for three hours at room temperature, plates were then washed six times with PBS and streptavidin-conjugated alkaline phosphatase (MABTECH) added for two hours. A further six washes were performed with PBS and cytokine-producing cells were detected after a 15min reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium using an alkaline phosphatase-conjugate substrate kit (Bio-Rad, Richmond, CA), and the reaction stopped by rinsing wells with tap water. Plates were then air-dried and the number of spot forming cells (SFC) was counted using an automated AID (Autoimmune Diagnostika GmbH) ELISPOT plate reader©. Negative control background values were subtracted from all responses. Negative values (background greater than response) were set to zero and classified as non-responders and analyses performed both excluding and including these values.

In phase II ELISPOT assays were performed at the WIMM, on thawed and rested (4hours) cryopreserved cells using a two-dimensional matrix of 21 peptide pools of known CD8+

restricted lytic and latent EBV epitopes identified in Caucasian donors (Table 2.4 and 2.5). Peptides, synthesised at the WIMM, were solubilised in DMSO at a stock concentration of 40mg/ml and stored at -20°C. Working dilutions in RPMI of 200µg/ml were used to create the pools giving a final concentration of peptide per well of 2µg/ml (Figure 2.6).

Table 2-4 *EBV Latent protein known CD8+ restricted epitopes identified in Caucasian donors*

LATENT proteins	Peptide ID	Epitope Sequence	Epitope Coordinate	HLA Restriction
EBNA3A	LA-1	QAKWRLQTL	158-166	B8
	LA-2	AYSSWMYSY	176-184	A30.02
	LA-3	RYSIFFDY	246-253	A24
	LA-4	FLRGRAYGL	325-333	B8
	LA-5	KRPPIFIRRL	378-387	B27
	LA-6	RPPIFIRRL	379-387	B7
	LA-7	LEKARGSTY	406-414	B62
	LA-8	HLAAQGMAY	450-458	-
	LA-9	YPLHEQHGM	458-466	B35.01
	LA-10	VFSDGRVAC	491-499	A29
	LA-11	VPAPAGPIV	502-510	B7
	LA-12	SVRDRLARL	596-604	A2
	LA-13	RLRAEAQVK	603-611	A3
	LA-14	VQPPQLTLQV	617-625	B46
EBNA3B	LA-15	HRCQAIRKK	149-157	B27.05
	LA-16	TYSAGIVQI	217-225	A24.02
	LA-17	RRARSLAERY	244-254	B27.02
	LA-18	VSFIEFVGW	279-287	B58
	LA-19	AVFDRKSDAK	399-408	A11
	LA-20	IVTDFSVIK	416-424	A11
	LA-21	AVLLHEESM	488-496	B35.01
	LA-22	VEITPYKPTW	657-666	B44
EBNA3C	LA-23	EGGVGWRHW	163-171	B44.03
	LA-24	QNGALAINTF	213-222	B62
	LA-25	LRGKWQRRYR	249-258	B27.05
	LA-26	RRIYDLIEL	258-266	B27
	LA-27	HHIWQNLL	271-278	B39
	LA-28	EENLLDFVRF	281-290	B44.02
	LA-29	LLDFVRFMGV	284-293	A2.01
	LA-30	LDFVRFMGV	285-293	B37
	LA-31	KEHVIQNAF	335-343	B44.02
	LA-32	FRKAQIQGL	343-351	B27.05
	LA-33	QPRAPIRPI	881-889	B7
LMP1	LA-35	FWLYIVMSD	38-46	-
	LA-36	FRRDLLCPLGA	72-82	B40
	LA-37	YLLEMLWRL	125-133	A2
	LA-38	YLQQNWWTL	159-167	A2
	LA-39	TLLVDLLWL	166-174	A2
	LA-40	DPHGPVQLSYYD	375-386	B51.1
LMP2	LA-41	MGSLEMVPM	1-9	B35.01
	LA-42	EDPYWGNDRHSDYQ	61-75	-
	LA-43	NPVCLPVIVAPYLF	121-134	-
	LA-44	LPVIVAPYL	125-133	B53
	LA-45	PYLFWLAAI	131-139	A23
	LA-46	ASCFTASVSTVVTA	141-154	-
	LA-47	FTASVSTVV	144-152	A68
	LA-48	IEDPPFNSL	200-208	B40.01
	LA-49	RRRWRLTV	236-244	B27.04
	LA-50	RRWRRLTVC	237-245	B14.02
	LA-51	RRLTVCGGIMF	240-250	B27
	LA-52	TVCGGIMFL	243-251	A2.01/06
	LA-53	MFLACVLVLIVDAV	249-262	-
	LA-54	LIVDAVLQL	257-265	A2
	LA-55	GLGTLGAAI	293-301	A2
	LA-56	LLWTLVVLL	329-337	A2.01
	LA-57	SSCSCPLSK	340-349	A11

LA-58	ILLARFLY	349-358	29
LA-59	FLYALALL	356-364	A2
LA-60	TYGPVFMCL	419-427	A24
LA-61	CLGGLTMV	426-434	A2.01
LA-62	VMSNTLLSAW	442-451	A25
LA-63	LTAGFLIFL	453-461	A2.06
LA-64	LLSAWILTA	447-455	A2

Table 2-5 *EBV Lytic protein known CD8+ restricted epitopes identified in Caucasian donors*

LYTIC proteins	Peptide ID	Epitope Sequence	Epitope Coordinate	HLA Restriction
BRLF1	LY-1	LVSDYCNVLNKEFT	25-39	B18
	LY-2	LVSDYCNVL	25-33	A2.05
	LY-3	DYCNVLNKEF	28-37	A24
	LY-4	AENAGNDAC	91-99	B45
	LY-5	IACPIVMRYYYVLDHLI	101-115	A24/C2
	LY-6	YVLDHLIVV	109-117	A2.01
	LY-7	FFIQAPSNRVMIPAT	121-135	-
	LY-8	ATIGTAMYK	134-142	A11
	LY-9	KHSRVRAYTYSKVLG	145-159	A3
	LY-10	RALIKTLPRASYSSH	225-239	A2
	LY-11	ERPIFPHPSKPTFLP	393-407	Cw4
	LY-12	QKEEAAICGQMDLS	529-543	B61
	LY-13	EVCQPKRIRPFHPPG	441-455	-
BZLF1	LY-14	LPEPLPQGQLTAY	52-64	B35.08
	LY-15	EPLPQGQLTAY	54-63	B35.01
	LY-16	APENAYQAY	81-89	B35.01
	LY-17	LQHYREVAA	101-115	C8
	LY-18	DSELEIKRYKNR	172-183	B18
	LY-19	RKCCRAKFKQLLQHYR	186-201	C6
	LY-20	RAKFKQLL	190-197	B8
	LY-21	SENDRLRL	209-217	B60
BMLF1	LY-22	KDTWLDARM	265-573	-
	LY-23	GLCTLVAML	280-288	A2.01
	LY-24	DEVEFLGHY	397-405	B18
	LY-25	SRLVRAILSP	435-444	B14
BMRF1	LY-26	CYDHAQTHL	20-28	A2
	LY-27	FRNLAYGRTCVLGKE	86-100	C3/C10
	LY-28	RPQGGSRPEFVKL	116-128	B7
	LY-29	TLDYKPLSV	208-216	A2.01
	LY-30	YRSGIIAVV	268-276	C6
	LY-31	LPLDLSVILF	286-295	B53
BAF0	LY-32	LLWAARPR		A2
BCRF1	LY-33	RRLVVTLQC	3-11	B27
BALF2	LY-34	ARYAYYLQF	418-426	B27
BILF2	LY-35	RRRKGWIP	240-248	B27
BLLF1 (gp350)	LY-36	VLQWASLAV		A2
BALF4 (gp110)	LY-37	FLDKGTYTL	276-284	A2
	LY-38	ILYNGWYA		A2
	LY-39	VPGETMCY		B35
	LY-40	APGWLIWTY		B35
BXLF2 (gp85)	LY-41	TLFIGSHVV		A2.01
	LY-42	SLVIVTTFV		A2.01
	LY-43	LMIPLINV		A2.01

(Hislop et al., 2007b)

	pool-11	pool-12	pool-13	pool-14	pool-15	pool-16	pool-17	pool-18	pool-19	pool-20	pool-21	PROTEIN
pool-1	LA-1	LA-2	LA-3	LA-4	LA-5	LA-6	LA-7	LA-8	LA-9	LA-10	LA-11	EBNA3A
pool-2	LA-12	LA-13	LA-14	LA-15	LA-16	LA-17	LA-18	LA-19	LA-20	LA-21	LA-22	EBNA3A/3B
pool-3	LA-23	LA-24	LA-25	LA-26	LA-27	LA-28	LA-29	LA-30	LA-31	LA-32	LA-33	EBNA3C
pool-4	X	LA-35	LA-36	LA-37	LA-38	LA-39	LA-40	LA-41	LA-42	LA-43	LA-44	LMP1/LMP2
pool-5	LA-45	LA-46	LA-47	LA-48	LA-49	LA-50	LA-51	LA-52	LA-53	LA-54	LA-55	LMP2
pool-6	LA-56	LA-57	LA-58	LA-59	LA-60	LA-61	LA-62	LA-63	LA-64	x	x	LMP2
pool-7	LY-1	LY-2	LY-3	LY-4	LY-5	LY-6	LY-7	LY-8	LY-9	LY-10	LY-11	BRLF1
pool-8	LY-12	LY-13	LY-14	LY-15	LY-16	LY-17	LY-18	LY-19	LY-20	LY-21	x	BRLF1/BZLF1
pool-9	LY-22	LY-23	LY-24	LY-25	LY-26	LY-27	LY-28	LY-29	LY-30	LY-31	LY-32	BMLF1/BMRF1/BAF0
pool-10	LY-33	LY-34	LY-35	LY-36	LY-37	LY-38	LY-39	LY-40	LY-41	LY-42	LY-43	BCRF1/BALF2/gp350/gp110/gp85

Figure 2-6 *Two dimensional matrix used illustrating peptide pools and proteins screened.*

Highlighted in red is an example of deconvolution of a positive single peptide IFN- γ response detected by reactivity to peptide pools 8 and 18. Peptides correspond to the codes defined in Tables 2.4 and 2.5. x indicates where an equal volume of the diluent was added containing no peptide.

PBMCs were screened against each of the 21-peptide pools with a final concentration of 2µg/ml of peptide per well. After the streptavidin-conjugated alkaline phosphatase (MABTECH) was added and washed off, 50µl per well of BCIP (5-bromo-4-chloro-3'-indolyphosphate)/NBT (nitro-blue tetrazolium) plus developer (Moss inc/ Europa Bioproducts MO711A) was added and plates were incubated for 15 mins and the reaction stopped by rinsing wells with tap water. Plates were air-dried and the number of SFC was counted using an automated AID (Autoimmune Diagnostika GmbH) ELISPOT plate reader© (Figure 2.7).

The visit one samples from 96 of 114 (84%) of the children were screened using the peptide pool matrix. To quantify the antigen-specific responses, background values from the negative control wells (DMSO) were deducted. Responses were regarded as positive if they were if >3 times the mean of the quadruplicate negative control wells per plate and ≥ 50 SFU/ 10^6 PBMCs. If negative background wells had ≥ 30 SFU/ 10^6 or the positive control wells were negative, had ≤ 30 SFU/ 10^6 , the assay would have been classified as 'failed'. This however was not the case for any of the donors screened (Figure 2.7).

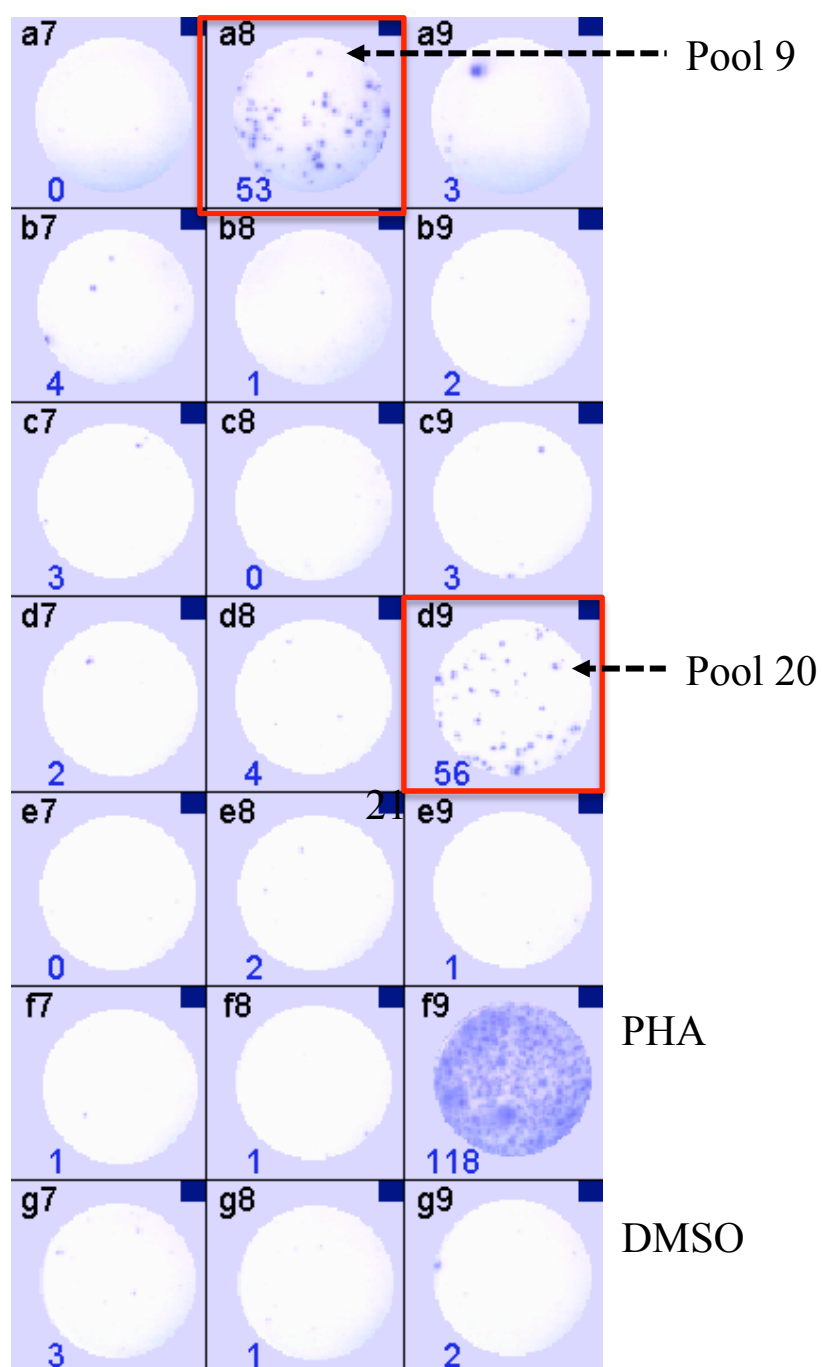


Figure 2-7 Example of donor ID044 screening positive to peptide pools 9 and 20 corresponding to epitope Ly-31 LPL

Cell numbers did not permit further screening of individual peptides when multiple wells responded. For further analysis in these instances it was not possible to determine which proportion of the response within the pool was attributable to which epitope, therefore these responses are presented as coming from either of the relevant proteins.

2.13 *Ex vivo* flow cytometric staining of human cells by the lysed whole blood method (Phase II)

T and B-cell surface antibody panels were used to directly stain whole blood (Table 2.6). For the B-cell antibody panel the whole blood was washed twice with PBS to remove any circulating immunoglobulin (Ig). A 100µl volume of whole blood for each donor was stained with the surface antibody panels for 30 min at 4°C. Red blood cells were then lysed using 1:10 FACS Lysing Solution (BD Biosciences) incubating for 10 min at room temperature. Cells were then washed twice in FACS buffer (PBS, 5% BSA, 5% EDTA) and re-suspended in Cytofix (BD, Biosciences). Samples were acquired on a Cyan™ ADP flow cytometer using Summit software (Beckman Coulter) at MRC Gambia. Compensation for fluorescence ‘spill-over’ was performed using the BD CompBead Anti-Mouse Ig set (BD Biosciences) and panel antibodies (Table 2.6). Briefly, antibodies were added to separate tubes containing one drop each of Anti-Mouse Ig beads and the negative control beads (which do *not* bind κ light chain-bearing immunoglobulin). Following a 30 min incubation at 4°C, beads were washed and re-suspended in FACS buffer. Analysis was performed using FlowJo (Tree Star Inc.) and isotype controls were used to set the gating for cell surface markers (Figure 3.0).

Table 2-6 Flow cytometry antibody panels used for *ex vivo*, intracellular and tetramer staining protocols using the CyAn flow cytometer.
Grey shading indicates an unused channel.

Channel	Laser (nm)	Phase I <i>Ex vivo</i> panel	Phase II <i>Ex vivo</i> whole blood T-cell panel	Phase II <i>Ex vivo</i> whole blood B-cell panel	Phase II Intracellular cytokine panel
FL-1	Argon 488	PD-1 FITC (BD Biosciences)	CD28 FITC (BD Biosciences)	IgD FITC (BD Biosciences)	Perforin FITC B-D48 (Gen-probe)
FL-2	Argon 488	Tetramers PE (A2, B35, B8)	CD3 PE (BD Biosciences)	CD21 PE (BD Biosciences)	IL-2 PE (Ebiosciences)
FL-3	Argon 488	CD28 ECD (Beckman Coulter)			CD8 qdot605 (Invitrogen)
FL-4	Argon 488	CD4 PerCP (BD Biosciences)	CD4 PerCP (BD Biosciences)	CD20 PerCP (BD Biosciences)	MIP1 β PerCP-Cy5.5 (BD Biosciences)
FL-5	Argon 488	CD19 PE-Cy7 (BD Biosciences)	CD62L PE-Cy7 (Ebioscience)	CD38 PE-Cy7 (BD Biosciences)	IFN γ PE-Cy7 (Ebioscience)
FL-6	Violet 405	CD8 PB (Ebioscience)	CD8 efluor450 (Ebioscience)	CD19 efluor450 (Ebioscience)	TNF α efluor450 (Ebioscience)
FL-7	Violet 405	CD3 Cas Yellow (Dako)			Live/Dead® Aqua (Invitrogen)
FL-8	Red 635	CD38 APC (BD Biosciences)	CD45RO APC (BD Biosciences)	IgM APC (BD Biosciences)	CD107a APC (BD Biosciences)
FL-9	Red 635	CD27 APC-alexafluor750 (Ebioscience)	CD27 APC-alexafluor750 (Ebioscience)	CD27 APC-alexafluor750 (Ebioscience)	CD4 efluor780 (Ebioscience)

FITC - Fluorescein Isothiocyanate, PE -Phycoerythrin, PerCP – Peridinin-chlorophyll-protein complex, PB – pacific blue, CY – cascade yellow, APC – Allophycocyanin, Cy –Cyanine dye, IL - Interleukin

2.14 Intra-cellular cytokine staining (ICS) following peptide stimulation (Phase II)

Overlapping BLZF1 and EBNA3A synthetic peptides

Overlapping peptide sets representing the entire EBV BZLF1 protein, a transcriptional activator that plays a critical role in the disruption of latency, and EBV EBNA-3A, essential for efficient immortalisation of B-cells, (n = 30 for BZLF1 and n = 117 for EBNA-3A) were used for peptide stimulation and ICS. In this study 18-mer peptides, overlapping by 10 amino acids were designed. Peptide sequences were based on EBV subtype 1. Overlapping peptides were designed using the Los Alamos National Library website's program PeptGen (<http://hiv-web.lanl.gov>) and synthesised by the PEPscreen services from Sigma-GenoSys (0.5-5mg, 70% purity).

Intra-cellular Cytokine Stimulation assay

Cells were aliquoted at 5×10^5 in 500µl R10 and stimulated with either an overlapping BZLF1 peptide pool or overlapping EBNA-3A peptide pool, both at 2µg/ml (each peptide), and co-stimulatory antibodies anti-CD28 and anti-CD49 (BD Bioscience, UK) at 1µg/ml each. Cells stimulated with *Staphylococcus* Enterotoxin B (SEB, Sigma) at 2µg/ml (positive control) and co-stimulatory antibodies alone (negative control) were included in the assay. Unstimulated cells were used for the unstained control and SEB-stimulated cells for fluorescence-minus-one (FMO) staining for perforin to aid with gating during flow cytometric analysis. Following incubation for one hour at 37°C, protein transport inhibitors Brefeldin A and Monensin (Ebioscience, UK, final concentrations 3µg/ml and 2µM respectively) and pre-titrated anti-CD107a-PE-Cy5 were added to stimulations. Following a further five hours incubation at 37°C, cells were washed using FACS wash buffer, supernatant discarded after

centrifugation, cells re-suspended in the residual volume and incubated for 10 mins in the dark at 4°C with LIVE/DEAD® fixable Aqua Dead Cell Stain (for 405nm excitation) (Invitrogen, UK). Surface stains with pre-titrated antibodies (Table 2.6) were then added and cells incubated for a further 30 mins at 4°C. Cells were then washed and fixed/permeabilised using Cytofix/Cytoperm™ (BD Biosciences). Briefly, 250µl Cytofix/Cytoperm™ was added and the sample incubated for 20 min at 4°C. Following permeabilisation, cells were washed with Perm/Wash™ buffer (BD Biosciences) and stained intra-cellularly with pre-titrated antibodies (Table 2.6) for 30 min at room temperature. Anti-perforin staining was excluded in the perforin-minus-one condition. Following staining, cells were washed twice in Perm/Wash™ buffer, once in FACS buffer and re-suspended in FACS buffer prior to analysis by flow cytometry within 12 hours.

Cells were analysed using a CyAn flow cytometer (Dako, Beckman Coulter). Flow cytometric compensation for fluorescence ‘spill-over’ was performed using the BD CompBead Anti-Mouse Ig, κ set (BD Biosciences) and antibodies used for cell staining (Table 2.6). Between 300,000 and 400,000 events were acquired in the lymphocyte gate for all samples. Data analysis was performed using FlowJo version 9.1 (Treestar, San Carlos, CA). Forward scatter (FSC)-area *versus* FSC-height parameters were used to exclude cell doublets, followed by exclusion of dead cells and subsequent gating on the lymphocyte population using FSC and side scatter (SSC) properties. CD4+ and CD8+ T-cells were then identified. For functional analysis, gates were created for each measured marker (CD107a, IFN-γ, tumour necrosis alpha (TNF-α), interleukin-2 (IL-2), macrophage inflammatory protein 1 beta (MIP1β)) and perforin). Boolean/combinatorial gate arrays were then created using FlowJo software, these determined the frequency of each possible response pattern based on all possible

combinations of the markers of interest, Figure 2.8 illustrates the gating strategy used. Responses detected in the negative (anti-CD28/anti-CD49d) control were subtracted from responses observed in stimulated samples. In cases where the value in the stimulated sample is smaller than that of the anti-CD28/anti-CD49d control, negative values arise following background subtraction. These were set to zero, but to avoid systematic bias with this approach, a threshold was set below which all positive values were also zeroed on the assumption that the distribution of background values is symmetric around zero (Roederer et al., 2011). This threshold was chosen based on the distribution of negative values for each dataset (CD8⁺ and CD4⁺ responses), by using the same absolute value as the 90th centile negative value. We detected a significant amount of CD107a background CD4⁺ and CD8⁺ T-cell staining in our unstimulated samples from Gambian children, Figure 2.8. This population of cells was excluded from further analysis, resulting in an analysis of 30 distinct functional populations as opposed to 31 populations. The BZLF1 cut off values were 0.09 and 0.04 for the CD8⁺ and CD4⁺ datasets respectively and 0.07 and 0.05 respectively for EBNA-3A.

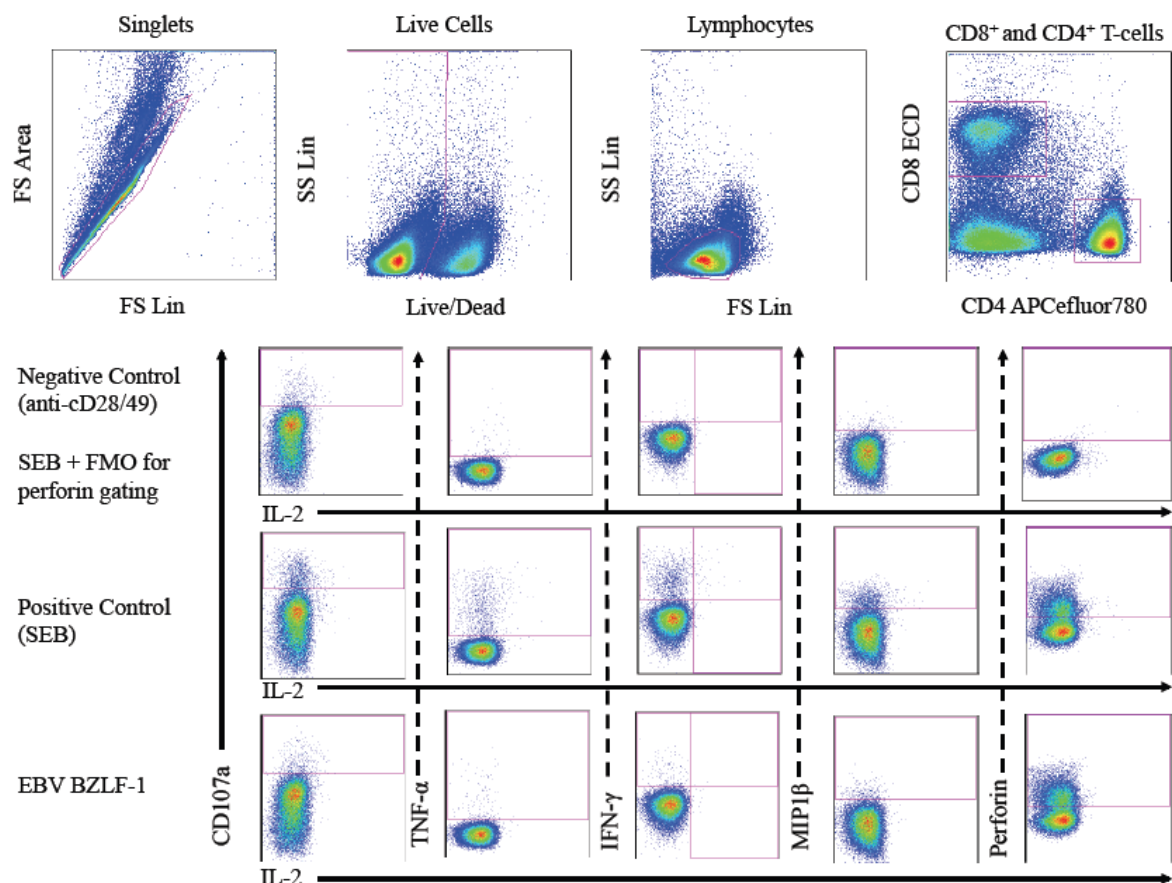


Figure 2-8 Plots from a representative donor illustrating flow cytometric gating strategy used for the intra-cellular cytokine staining following stimulation with EBV BZLF-1 in Phase II. Plots shown demonstrate staining on the total CD8⁺ T-cell population, with negative controls and fluorescence minus one (FMO) used for setting the gates.

2.15 Tetramer Synthesis

The tetramers used for phase I were as follows: A2-CLGGLLTMV (LMP2), A2-GLCTLVAML (BMLF1), B35-EPLPQGQLTAY (BZLF1) and B8-RAKFKQLL (BZLF1). All except A2-CLGGLLTMV (LMP2) were also used in phase II.

A2-GLCTLVAML (BMLF1) and B35-EPLPQGQLTAY (EBV BZLF1) class I tetramers were synthesised using the following procedure. A. Hislop from the Institute for Cancer Studies in Birmingham donated HLA-A2 and HLA_B35 MHC Class I heavy chains and β 2-

microglobulin cloned into vectors. These proteins were amplified and purified from bacterial inclusion bodies and solubilised in eight molar urea. These were refolded with the appropriate epitopes A2-GLCTLVAML (BMLF1) and B35-EPLPQGQLTAY, biotinylated and purified by Fast Protein Liquid Chromatography (FPLC). The monomeric complexes (50µg) of peptide/MHC class I, β 2-microglobulin and biotin were freshly conjugated to streptavidin-PE (Sigma-Aldrich) at 4:1 molar ratio to produce tetrameric peptide-MHC complexes. The remaining class I tetramers used in this work were produced by Yanchun Peng at the WIMM, except for an A2-GLCTLVAML (BMLF1) conjugated to streptavidin-APC that was provided by Heather Long from the Institute for Cancer Studies in Birmingham. Tetramers were tested against a seronegative donor and two seropositive donors, HLA matched and mismatched prior to use on samples (Figure 2.9).

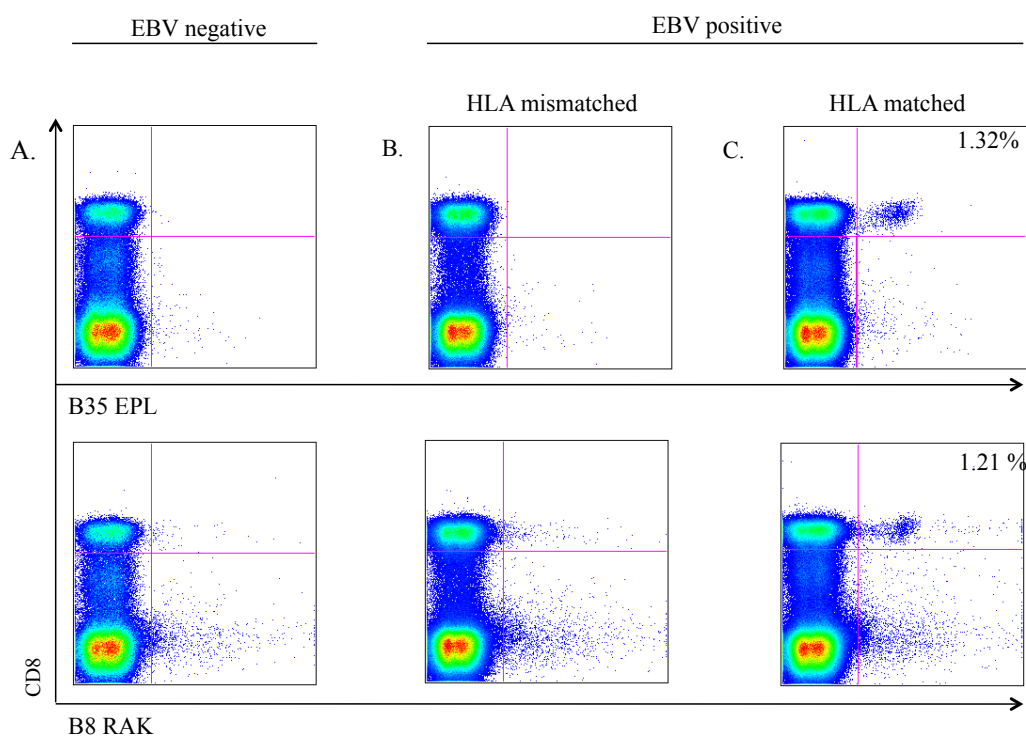


Figure 2-9 Example of tetramer staining for B35 EPLPQGQLTAY (BZLF1) and B8 RAKFKQLL (BZLF1) on seronegative (A), seropositive HLA mismatched (B) and HLA matched (C) donors.

Percentage indicates tetramer percentage as a proportion of CD8+ T-cell population.

2.16 Tetramer staining assays

For Phase I *ex vivo*, 1×10^6 PBMCs per MHC Class I tetramer (for lower cell counts all four tetramers were pooled) were stained for one hour at 4°C in the dark. MHC class I Tetramers containing GLCTLVAML (A2_GLC), CLGGLTMV (A2_CLG), EPLPQGQLTAY (B35_EPL) and RAKFKQLL (B8_RAK) peptides were used, conjugated to PE (A2_GLC synthesised at the Institute for Cancer Studies, University of Birmingham, the others from the WIMM, Oxford). Tetramer staining was followed by surface marker staining (30 min, 4°C in the dark) using pre-titrated surface antibodies (Table 2.6, Phase I *ex vivo* panel). Appropriate isotype control antibodies, IgG1 FITC, IgG1 PerCP, IgG1 PE-Cy7, IgG1 APC, (BD Biosystems) IgG2a Pacific Blue, IgG1 APC-Alexa750, (Ebioscience) and IgG1 Cascade Yellow (Dako), were used. For Phase I a set of compensation tubes was run for each donor using donors cells and the same antibodies as the panel where possible. Samples were acquired on a Cyan™ ADP flow cytometer using Summit software (Beckman Coulter) at MRC Gambia. Analysis was performed using FlowJo (Tree Star Inc.). FSC-area and FSC-height were used to exclude cell aggregates, followed by gating on CD3+ cells. FSC and SSC were then used to gate on lymphocytes, followed by isolation of CD8+ cells and EBV-specific tetramer-positive cells for further gating to examine expression of specific markers with isotype controls used for gating (Figure 2.10).

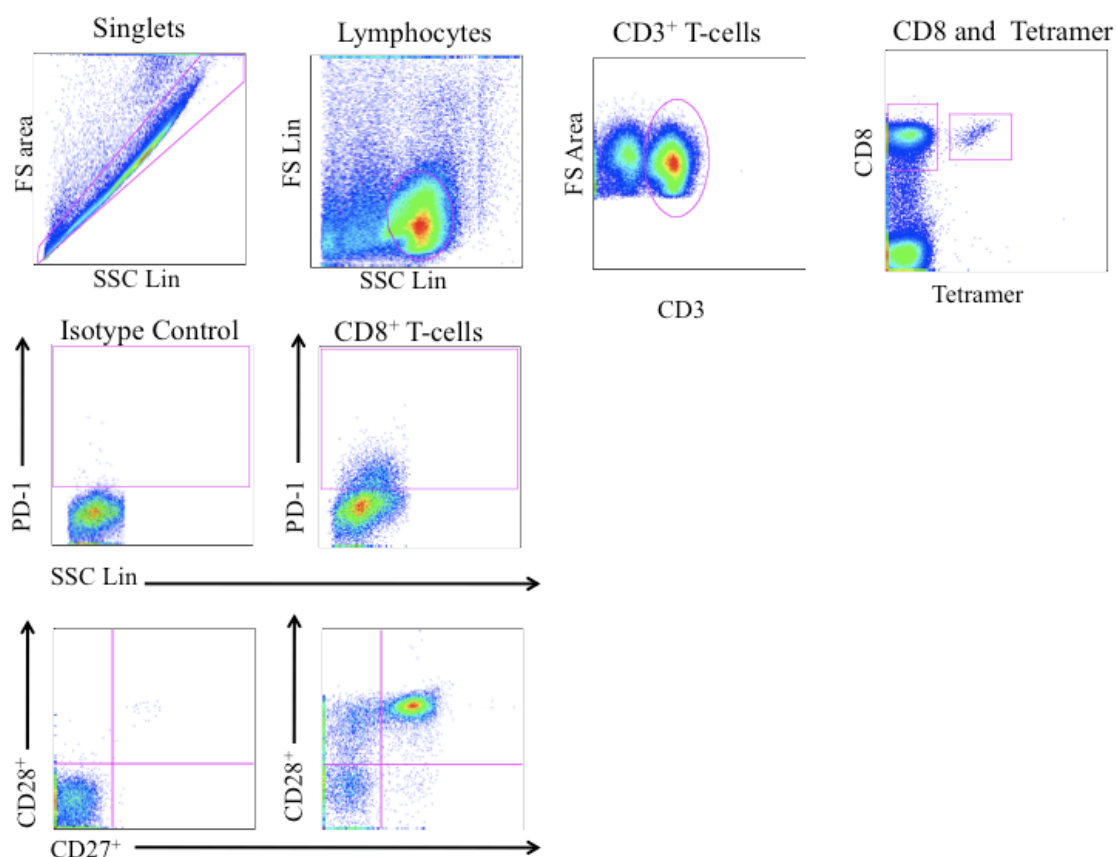


Figure 2-10 *Flow cytometry plots from a representative donor illustrating gating strategy used in Phase I.*

Plots demonstrate gating used to identify CD8+ and Tetramer positive populations and use of isotype controls for gating on total CD8+ population. These gates were then applied to EBV-specific Class I tetramer positive cells.

In Phase II, tetramer staining was performed on cryopreserved cells (see Table 2.7 for antibody panels used). Unconjugated CD57 monoclonal antibody (mAb) (clone NK-1, BD Biosciences) was conjugated in-house by Dr. T de Silva to Quantum dot 565 stain obtained from Invitrogen according to the manufacturer's protocol. The conjugated antibody was stored at 4°C prior to further use. All antibodies had been pre-titrated by Dr. T de Silva in an iterative process to obtain optimum volumes required for flow cytometric staining, until full panels to be used in final experiments were complete.

The cells were thawed (Section 2.11) and then stained with LIVE/DEAD® fixable Aqua Dead Cell Stain for 30 min at 4°C, washed and stained with 1µg of tetramer-PE for 15min at 37°C. Following two further washes, surface staining with CD3-Qdot655, CD4-Qdot605, CD8-Qdot705, CD14-V500, CD19-V500, CD27-efluor780, CD45RO-PETexasRed, CD57-Qdot565 and PD-1-PE-Cy7 were performed in all test samples. Additional surface staining with 2B4-PE-Cy5.5 and CD160-efluor647 in one panel (i), and CD38-APC and HLA-DR-Alexafluor700 in another panel (ii) were performed. Following fixing and permeabilisation as described above, intracellular staining with Perforin-FITC, Granzyme B-Alexafluor700 and T-bet (T-box transcription factor) -Brilliant Violet in panel (i) and Ki67-Alexafluor488 and Bcl-2 (B-cell lymphoma 2) -V450 in panel (ii) was performed. FMO samples were included to aid gating during subsequent flow cytometric analysis.

Table 2-7 Antibody panels used on the LSRII.
Grey Shading indicates an unused channel.

Detector	Laser	Phase II Tetramer Panel (i)	Phase II Tetramer Panel (ii)
B710-A	Blue 488		Ki67 alexafluor488 (BD Biosciences)
B515-B	Blue 488	Perforin (D48) FITC (Diaclone)	
G780-A	Green 532	PD-1 PE-Cy7 (Biolegend)	PD-1 PE-Cy7 (Biolegend)
G710-B	Green 532	2B4 PE-Cy5.5 (Biolegend)	CD4 PE-Cy5.5 (Invitrogen)
G610-D	Green 532	CD45RO PE-Texas Red (Invitrogen)	CD45RO PE-Texas Red (Invitrogen)
G560-E	Green 532	Tetramers PE (A2, B35, B8)	Tetramer PE (A2, B35, B8)
R780-A	Red 633	CD27 APCeFluor780 (Ebioscience)	CD27 APCeFluor780 (Ebioscience)
R710-B	Red 633		HLA-DR alexafluor700 (Biolegend)
R660-C	Red 633	CD160 APC (Biolegend)	CD38 APC (Biolegend)
V705-A	Violet 407	CD8 Q705 (Invitrogen)	CD8 Q705 (Invitrogen)
V655-B	Violet 407	CD3 Q655 (Invitrogen)	CD3 Q655 (Invitrogen)
V605-C	Violet 407	CD4 Q605 (Invitrogen)	
V565-E	Violet 407	CD57 Q565 (in-house conjugation)	CD57 Q565 (in-house conjugation)
V505-G	Violet 407	CD14/CD19 V500 Live/Dead® Aqua (Invitrogen)	CD14/CD19 V500 Live/Dead® Aqua (Invitrogen)
V450-H	Violet 407	T-bet Brilliant Violet (Biolegend)	Bcl-2 V450 (BD Biosciences)

FITC - Fluorescein Isothiocyanate, PE -Phycoerythrin, APC - Allophycocyanin

Compensation for fluorescence ‘spill-over’ was performed using the BD CompBead Anti-Mouse Ig set (BD Biosciences) and antibodies as described above. Between 500,000 and 800,000 events were acquired for each sample. Data analysis was performed using FlowJo version 9.1 (Treestar). FSC-area and FSC-height were used to exclude cell aggregates, followed by removal of CD14⁺, CD19⁺, dead cells and gating on CD3⁺ cells. FSC and SSC were then used to gate on lymphocytes, followed by isolation of CD8⁺ cells and tetramer-positive cells for further gating to examine expression of specific markers (Figure 2.11 and 2.12). Similar approaches have been shown to reduce background staining during detection of tetramer-positive populations (Chattopadhyay et al., 2008).

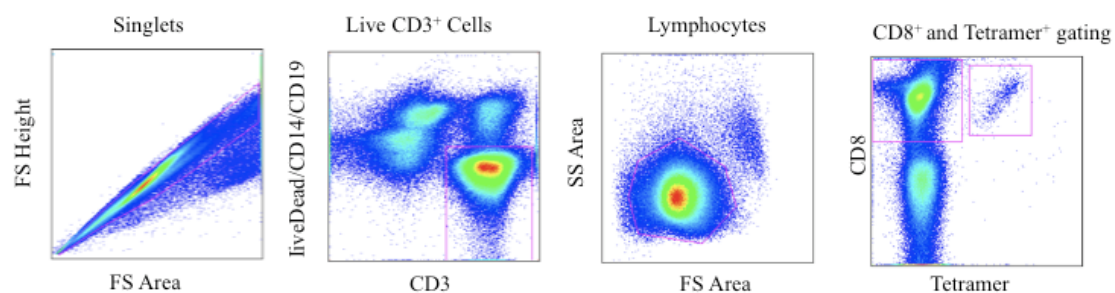


Figure 2-11 Representative donors illustrating gating strategy used to identify EBV-specific Class I tetramer positive cells and CD8⁺ T-cells.

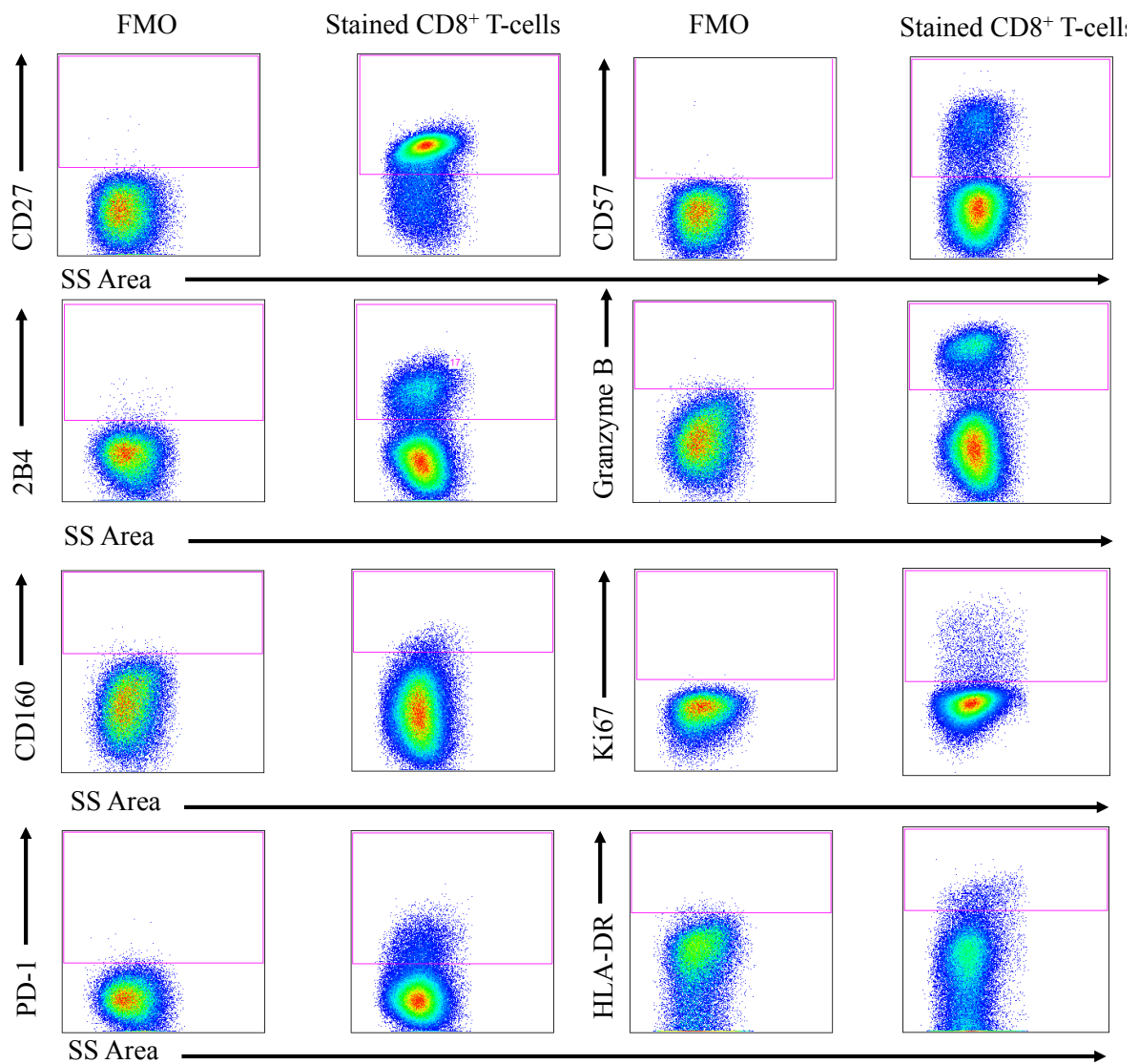


Figure 2-12 Representative donor illustrating setting of gates using fluorescence minus one samples applied to total CD8⁺ T-cell populations.
Gates were then copied and applied to EBV-specific Class I tetramer positive cells.

2.17 Statistics analyses

Viral genome load, serology, ELISPOT and flow cytometry data were plotted and statistically analysed using GraphPad Prism software (Version 5; GraphPad). Wilcoxon rank and Mann-Whitney U non-parametric tests were used for comparisons of matched and unmatched data respectively. STATA (version 11) was used to calculate Spearman's rank correlation coefficients for Phase I.

All statistical analyses and graphical presentations in Phase II were performed using Microsoft Excel for Macintosh 2011 and Graphpad Prism version 5.0 for Macintosh (GraphPad Software, San Diego California, USA, www.graphpad.com). Comparisons between variables were performed using the Mann-Whitney U test (two-tailed) and Wilcoxon matched pair test (for comparisons between total CD8⁺ and virus-specific CD8⁺ T-cells made within individuals). Correlations between non-normally distributed data were made using the Spearman's rank correlation coefficient.

CHAPTER 3 : THE EFFECT OF ACUTE *P. FALCIPARUM* MALARIA ON EPSTEIN-BARR VIRUS: HOST BALANCE IN THE SETTING OF REDUCED MALARIA ENDEMICITY

3.1 Introduction

EBV first colonises the B-cell system through a growth-transforming infection, where coordinate expression of specific latent cycle genes drives the expansion of infected B-cell clones. These expansions are controlled by the EBV-specific T-cell response but it appears that some infected cells survive by extinguishing latent gene expression; thereby generating a reservoir of latently infected resting B-cells, through which the virus persists (Babcock et al., 2000). Reactivations from this reservoir do nevertheless occur and, if not controlled by virus-specific T-cells, can lead to further B-cell transformation events and greatly increased loads of latently-infected B-cells in the blood (Speck et al., 1997). In the West, this is most apparent in heavily T-cell suppressed transplant patients, where high circulating EBV loads often precede the appearance of EBV-positive PTLN (Rezk and Weiss, 2007, Rickinson, 2002). A somewhat analogous situation is thought to occur in Africa, where the similar geographic distributions of eBL, an EBV-associated childhood B-cell tumour, and holoendemic malaria suggests that malaria may predispose to eBL through suppression of EBV-specific T-cell surveillance leading to increased viral loads (Rochford et al., 2005).

Several studies conducted in areas with high malaria endemicity have indeed implied that malaria exposure can cause a reduction in EBV-specific T-cell responses (Whittle et al., 1984). Early assays quantifying the regression of EBV-infected B-cell outgrowth in PBMC cultures, an effect mediated by *in vitro* reactivation of EBV latent antigen-specific CD8+

cytotoxic T-cells (Gudgeon et al., 2005), showed a slight relaxation of T-cell control in healthy adults from holoendemic malarial versus non-endemic malarial areas of New Guinea (Moss et al., 1983). Subsequently, a study of healthy children in Kenya with differential malaria exposure has shown that a smaller proportion of children, five to nine years of age, had EBV-specific IFN- γ ELISPOT responses (PBMC), when living in malaria-holoendemic areas, compared to those living in areas where exposure is only sporadic (Moormann et al., 2007). EBV genome loads in whole blood of these children were also significantly higher in the holoendemic malaria group, but only in children one to four years of age (Moormann et al., 2005). Turning to acute malaria itself, T-cell control as measured by the regression assay appeared to be completely abrogated in children from an area at the time considered to be holoendemic, The Gambia, during an episode of acute malaria, but recovered to some extent after treatment and the resolution of symptoms (Whittle et al., 1990, Whittle et al., 1984). More recently EBV-specific CD8⁺ T-cell responses restricted through two HLA alleles, B*3501 and B*5301, were measured by IFN- γ ELISPOT in Gambian children with acute malaria and compared to results obtained up to six weeks post-treatment. With the caveat of a small sample size in mind, this comparison suggested that responses were impaired during acute disease, but it was not clear whether this reflected a reduction in the numbers or in the functional competence of EBV-specific T-cells in the circulation (Njie et al., 2009). A further heterogeneous group of studies have looked at EBV viral load dynamics during malaria exposure (Donati et al., 2006, Rasti et al., 2005, Yone et al., 2006). Comparability of these results is limited, as the assays have been conducted on differing sample types, either whole blood, plasma alone or PBMC preparations. Yone *et al.* saw significantly higher whole blood viral loads in acute uncomplicated malaria cases compared to convalescence, with a similar non-significant trend for complicated malaria cases (Yone et al., 2006). In The Gambia,

PBMC EBV genome loads did not differ between acute and convalescent malaria cases, but were elevated in the cases compared to healthy controls (Njie et al., 2009).

Since the original observations of impaired EBV control in The Gambian population in a setting of holoendemic malaria were made in the 1980's (Whittle et al., 1984) the intensity of malaria infection had reduced substantially in this geographical region (Satoguina et al., 2009, Ceesay et al., 2008, Ceesay et al., 2010). This study examines immune control of EBV in this setting of reduced malaria exposure by studying T-cell function using regression assays. More contemporary immune measures of MHC Class I tetramer staining and IFN- γ ELISPOT analysis in children during acute malaria infection and on convalescence were also included to assess numbers and functional competence of EBV-specific CD8⁺ T-cells.

3.2 Methods

3.2.1 Study Design and Donors

Children were serially recruited in The Gambia during the 2008/9 malaria season infected with acute uncomplicated *P. falciparum* malaria defined as a temperature $>37.5^{\circ}\text{C}$ within the last 48 hours, and ≥ 5000 parasites/ μl detected by slide microscopy. Samples were obtained from MRC Fajara outpatient clinic and the Jammeh Foundation for Peace Hospital in Bundung. Assays were conducted in real time during acute (Day 0) and convalescent (Day 28) stages of the *P. falciparum* infection in children aged 1-15 yrs. Healthy age- and sex-matched control children without malaria were recruited during the subsequent dry season (May 2009). Chapter 2, section 2.1 and 2.2 details sites, samples taken and processing in depth. Matching of controls was performed according to sex- and age- by identifying appropriate children using data from a demographic survey conducted in Brefet by a previous PhD student (O. Finney). Children were then sought out by field-workers and recruited into the study until sufficient numbers of control patients were reached.

3.2.2 Sickle cell trait diagnosis

Sickle cell status was determined by metabisulfite test and a positive result was confirmed by cellulose acetate electrophoresis to determine the exact genotype (Hicksg et al., 1973). The MRC Gambia Haematology Laboratories performed this, in addition to a FBC on all study participants.

3.2.3 Malaria Diagnosis by Slide

Thick blood films were prepared with 10µl of blood, and stained with Giemsa following standard methods. At the health clinics, 50 high power fields (HPF) for each thick film from the malaria cases were screened. These were then confirmed on a thin film by reading of a further 100 HPF under oil emersion (1000x). For the controls 200 HPF for each thick film were screened. Malaria PCR was also performed on all donors (Methods section 2.7).

3.2.4 EBV Genome loads and Cellular Immunological Assays

EBV viral genome loads were performed on DNA extracted from 500µl whole blood see Methods Chapter 2, section 2.6. EBV-specific CD8⁺ T-cell responses were assessed using two functional assays (regression and IFN- γ ELISPOT) that had been used separately in earlier work (Njie et al., 2009, Whittle et al., 1984), and numbers of EBV epitope-specific cells were enumerated by HLA class I-peptide tetramer staining. Laboratory methods used are detailed in Methods Chapter 2, Sections 2.12, 2.15 and 2.16.

3.3 Results

3.3.1 Demographics and characteristics of study populations

Table 3.1 shows that the two groups of infected and uninfected children were similar with regard to age and sex, indicating successful matching. None of the study participants were found to be Sick Cell positive on screening. As expected there was a significant decline in haemoglobin levels during the acute phase of malaria, which on convalescence is not significantly different to that found in uninfected controls. Leucocyte counts did not significantly differ from convalescent or uninfected matched control samples during acute *P. falciparum* infection. The percentage of lymphocytes was significantly higher on convalescence, at which time it did not significantly differ from uninfected controls. This is consistent with what has been previously described (Lisse et al., 1994).

Table 3-1 Characteristics of uncomplicated *P. falciparum* cases and controls

	Acute <i>P.falciparum</i>	Convalescent <i>P.falciparum</i>	Aparasitaemic Controls	Acute versus Convalescent ¹	Convalescent versus Controls ²
Age (yrs)	7 (4.8-10.0)	-	7 (4.5-9.5)	-	0.95
Male: Female Ratio	1.91	-	2.00	-	-
Haemoglobin (g/dl)	11.25 (9.83-12.63)	12.50 (11.40- 12.93)	12.40 (11.65-13.20)	0.0038	0.65
Leucocyte Count (x10 ⁹ /L)	6.96 (4.90-11.13)	6.40 (5.20-8.90)	6.10 (5.30-7.70)	0.31	0.87
Lymphocytes (%)	33.35 (22.30-41.83)	52.30 (46.90- 56.93)	51.45 (45.23-63.30)	<0.0001	0.92
Lymphocyte Count (x10 ⁹ /L)	2.00 (1.53-2.75)	3.25 (2.68-4.90)	3.15 (2.60-4.35)	0.0004	0.72

For age and haemoglobin levels the median value and inter-quartile range are shown in brackets.

¹ p value calculated using Wilcoxon rank statistical comparison of matched pairs

² p value calculated using Mann-Whitney U statistical comparison of unpaired data

Of the *P. falciparum* infected children 84% had at least one of the HLA alleles of interest (A2, B35, B8, B53), as did 81% of the control children. The HLA types detected were of a similar frequency in both case and control groups (Figure 3.1).

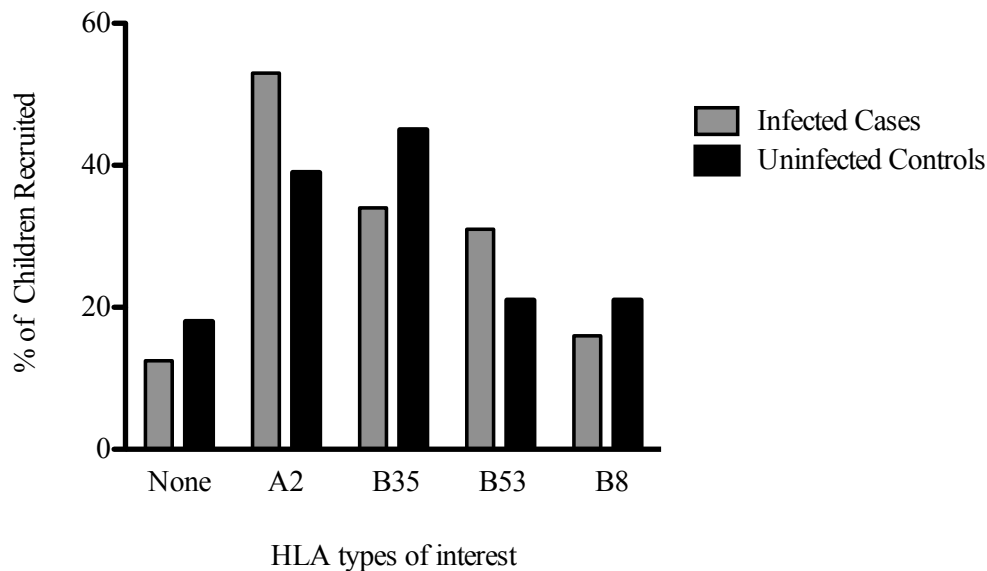
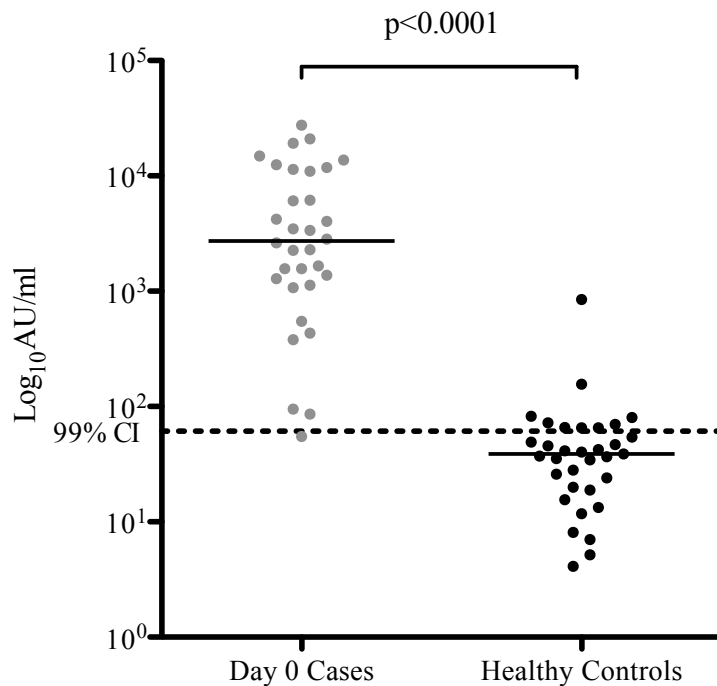


Figure 3-1 Percentage of children with HLA types of interest in cases (n=32) and controls (n=33).

All malaria cases were both slide and PCR positive with a geometric mean slide parasite density of 135,878/ μ l (95% CI 91,194 – 202,456). All controls were slide and PCR negative for *P. falciparum* parasites. In order to establish whether the control children had undergone a recent *P. falciparum* infection, ELISAs were performed to MSP-1(19) on both the infected (Day 0) and uninfected control children. The uninfected children had significantly lower anti-MSP-1(19) antibody levels, when compared to the *P. falciparum* infected cases (Figure 3.2). Using the upper limit of the 99% Confidence Interval of values obtained from 20 non-exposed individuals as a cut off, on Day 0, 97% of infected children were classified as seropositive compared to 24% of the control children.

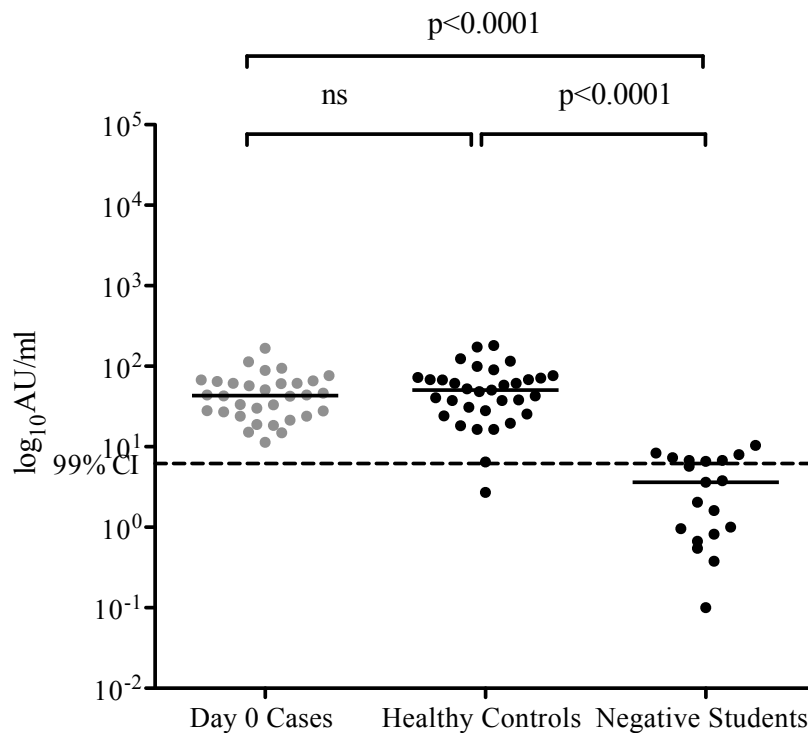


P value calculated using Mann-Whitney U comparison of unpaired data.

Figure 3-2 Comparison of the magnitude of *P. falciparum* merozoite surface protein-1(19), (MSP-1(19)), antibody levels in acute uncomplicated *P. falciparum* cases (n=32) and healthy controls (n=33).

Solid lines indicate median values and the dotted line indicates the cut off value for positivity, the upper limit of the 99% confidence interval obtained from sera from 20 non-exposed individuals.

All of the malaria cases and all except one of the uninfected controls, who was excluded from further analysis, were EBV positive by IgG VCA ELISA (Figure 3.3). Comparisons were made to a group of EBV naïve university students as an additional control as described in the Methods section 2.8.1.



P value calculated using Mann-Whitney U comparison of unpaired data.

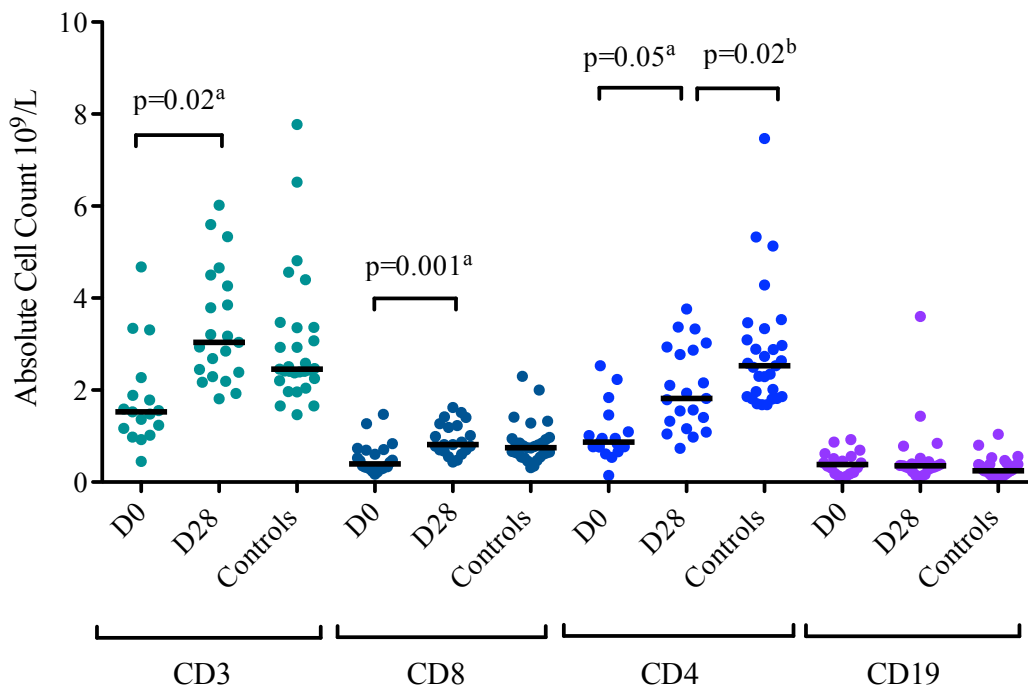
Figure 3-3 Comparison of the magnitude of EBV VCA IgG levels in acute uncomplicated *P. falciparum* cases (n=32), healthy controls (n=33) and EBV uninfected students (n=19).

Solid lines indicate median values and the dotted line indicates the cut off value for positivity, the upper limit of the 99% confidence interval obtained from plasma from 19 uninfected students.

3.3.2 Frequencies of total cellular populations in *P.falciparum* infected and uninfected children

Absolute total CD4+ and CD8+ T-cell counts were significantly lower during acute (Day 0) *P. falciparum* infection compared to convalescence (Day 28) consistent with previous findings (Lisse et al., 1994, Walther et al., 2009). The total number of B-cells however did not significantly differ between acute and convalescent samples or between infected (Day 0 or

Day 28) and uninfected control children (Figure 3.4). The number of CD19+CD27+, memory B-cells, also did not significantly differ between acute, convalescent and uninfected control samples with median values of $0.16 \times 10^9/L$ (0.08-0.31), $0.12 \times 10^9/L$ (0.07-0.22) and $0.16 \times 10^9/L$ (0.11-0.27) respectively.



^a *P* value calculated using Wilcoxon rank statistical comparison of matched pairs.

^b *P* value calculated using the Mann-Whitney U comparison of unpaired data.

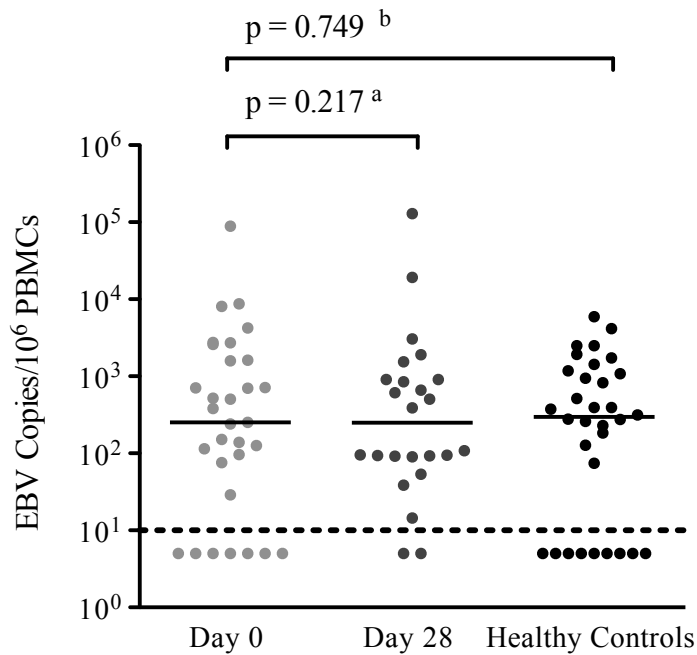
Figure 3-4 Total cellular frequencies in *P. falciparum* infected and uninfected children.

There is a significant decrease in the numbers of CD3, CD8 and CD4 positive cells in acute uncomplicated *P. falciparum* malaria (D0), compared to cell numbers during convalescence in the same donors (D28). The number of CD4 positive cells on convalescence remains significantly lower than age- and sex- matched healthy controls. The number of CD19 positive B-cells does not significantly differ.

3.3.3 EBV genome loads did not significantly change during acute uncomplicated *P. falciparum* malaria infection

In contrast to previous findings documented in the literature (Njie et al., 2009), no differences were found in EBV genome loads between children with acute (Day 0) *P. falciparum* malaria and age- and sex- matched healthy controls ($p=0.749$). Likewise, the EBV genome loads did not change significantly between D0 and D28 ($p=0.2173$) (Figure 3.5). Considering the number of B-cells remained constant (Figure 3.4) it is unlikely that an increase in the total number of B-cells would have obscured an increase in total EBV genome load. There were 7, 2, and 9 donors with undetectable viral loads in acute (D0), convalescent (D28) and control groups respectively, although all had a positive EBV serology.

Consistent with previous findings (Moormann et al., 2005, Njie et al., 2009) there was a significant inverse correlation between EBV genome load and age in the uninfected controls and cases during convalescence (Figure 3.6). With the caveat of a small sample size, the analysis of these data looking at subgroups of different age ranges (0-7yrs and 8-15yrs) also showed no significant differences in EBV genome loads between children with acute (Day 0) *P. falciparum* malaria and age- and sex- matched healthy controls. Likewise, the EBV genome loads did not change significantly between D0 and D28 (Figure 3.7).

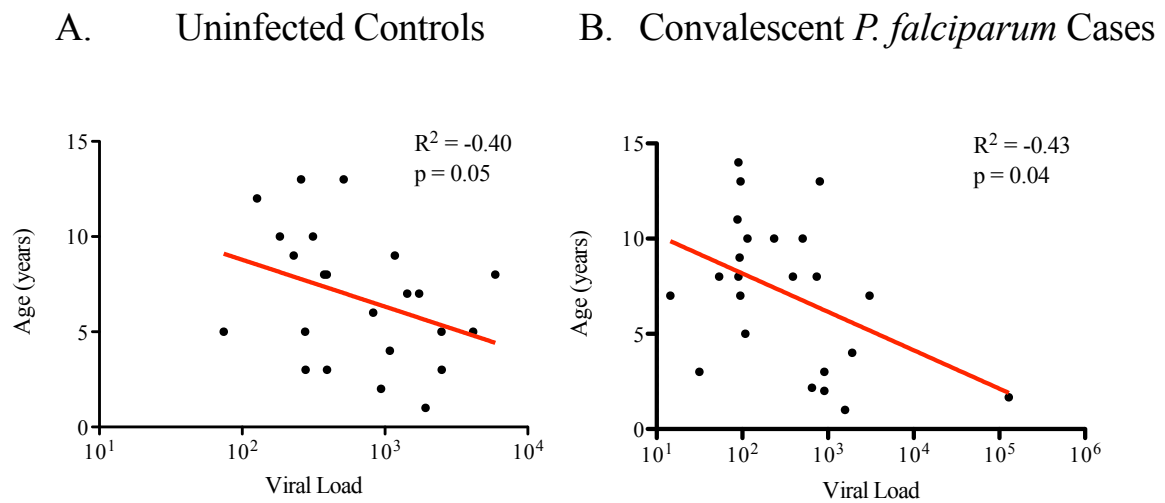


^a *P* value calculated using Wilcoxon rank statistical comparison of matched pairs.

^b *P* value calculated using the Mann-Whitney U comparison of unpaired data.

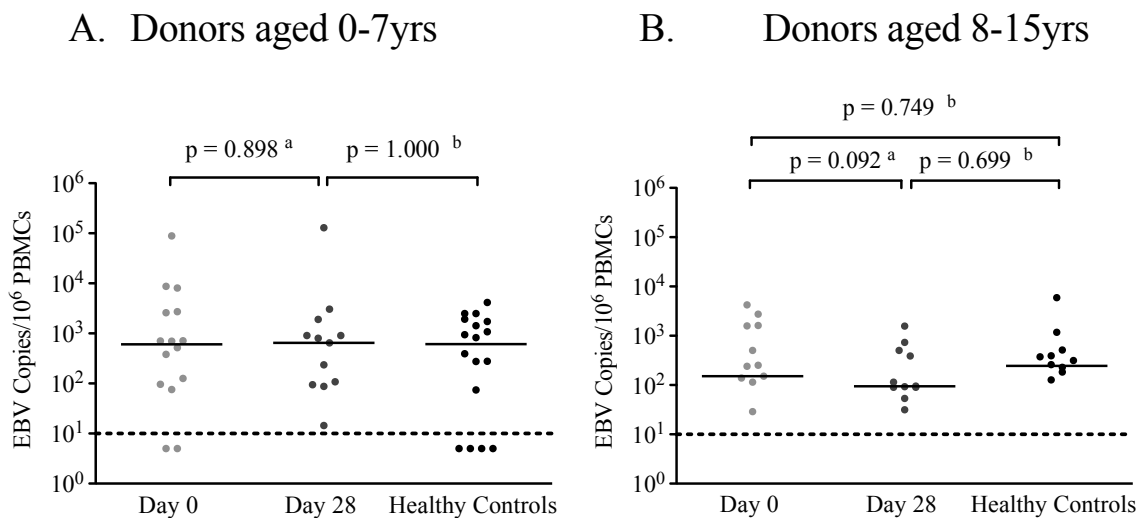
Figure 3-5 *EBV genome loads during acute uncomplicated *P. falciparum* malaria infection (*n* = 31), on convalescence in the same donors (*n* = 24), and from healthy age- and sex-matched controls (*n* = 32).*

The median values, illustrated by the solid black lines, for the groups were 253, 249 and 296 EBV copies/10⁶ PBMCs respectively. The dashed line represents the lower limit of detection for EBV genomes in the assay. Donors below the dashed line had undetectable viral loads



Spearman correlation co-efficient was calculated excluding values below the limit of detection of the assay.

Figure 3-6 Detectable EBV genome loads declined with age in uninfected controls (A) and *P. falciparum* infected cases at convalescences (B).



^a P value calculated using Wilcoxon rank statistical comparison of matched pairs.

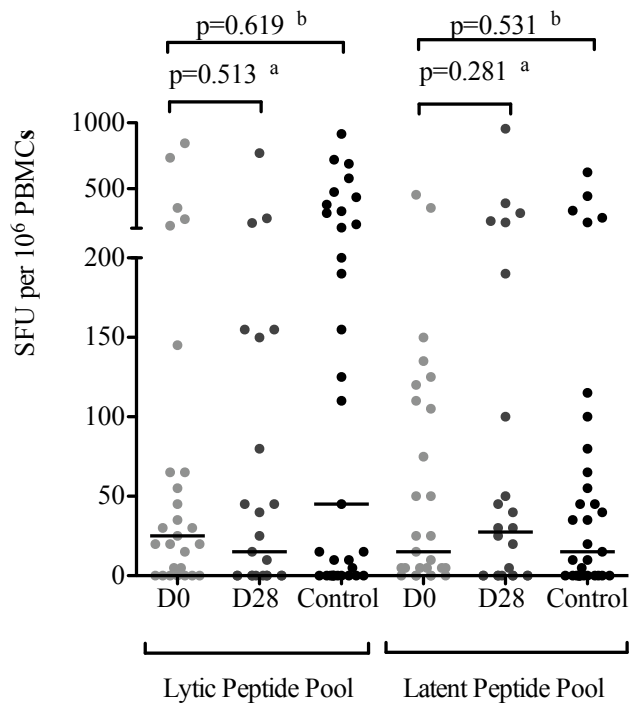
^b P value calculated using the Mann-Whitney U comparison of unpaired data.

Figure 3-7 EBV genome loads during acute uncomplicated *P. falciparum* malaria infection, on convalescence in the same donors, and from healthy age- and sex- matched controls in children age 0-7years (A) and 8-15years (B).

The solid black lines illustrate the median values and the dashed line represents the lower limit of detection for EBV genomes in the assay.

3.3.4 EBV-specific T-cell IFN- γ ELISPOT responses to lytic and latent CD8+ restricted epitopes did not significantly change during acute uncomplicated *P.falciparum* infection

Of the of case and control donors assessed respectively, 84% and 81% had appropriate HLA types for the EBV lytic and latent epitope peptide pools screened. For the EBV lytic peptide pools there were 7 (29%), 7 (30%) and 8 (31%) non-responders in the acute (Day 0), convalescent (Day 28) and control groups, respectively. Numbers of non-responders were of a similar magnitude for the EBV latent epitope peptide pool, being 6 (25%), 9 (39%) and 8 (31%) for acute (Day 0), convalescent (Day 28) and control groups, respectively. Background values were of a similar magnitude in all groups, with medians of 15 SFU/10⁶ PBMCs in Day 0, 28 and control groups. Consistent with the observations made for viral loads, EBV-specific IFN- γ ELISPOT counts did not differ between children with acute (Day 0) *P. falciparum* malaria and age- and sex- matched healthy controls to both EBV lytic (p=0.6191) and latent (p=0.5305) epitope peptide pools. Likewise, there was no significant difference between Day 0 and Day 28 to both EBV lytic (p=0.5129) and latent (p=0.2808) epitope peptide pools when negative values (non-responders) were counted as zero (Figure 3.8; see Methods Section 2.13). The results did not alter when all non-responders were excluded from the analysis, when analysed without deduction of the background values and when analysed taking into account the difference in CD8% between Day 0 and Day 28. The CD8% at Day 0 and Day 28 as measured by flow cytometry was used to calculate the SFU per 10⁶ CD8⁺ T-cells from the values detected for total PBMCs (Figure 3.9).

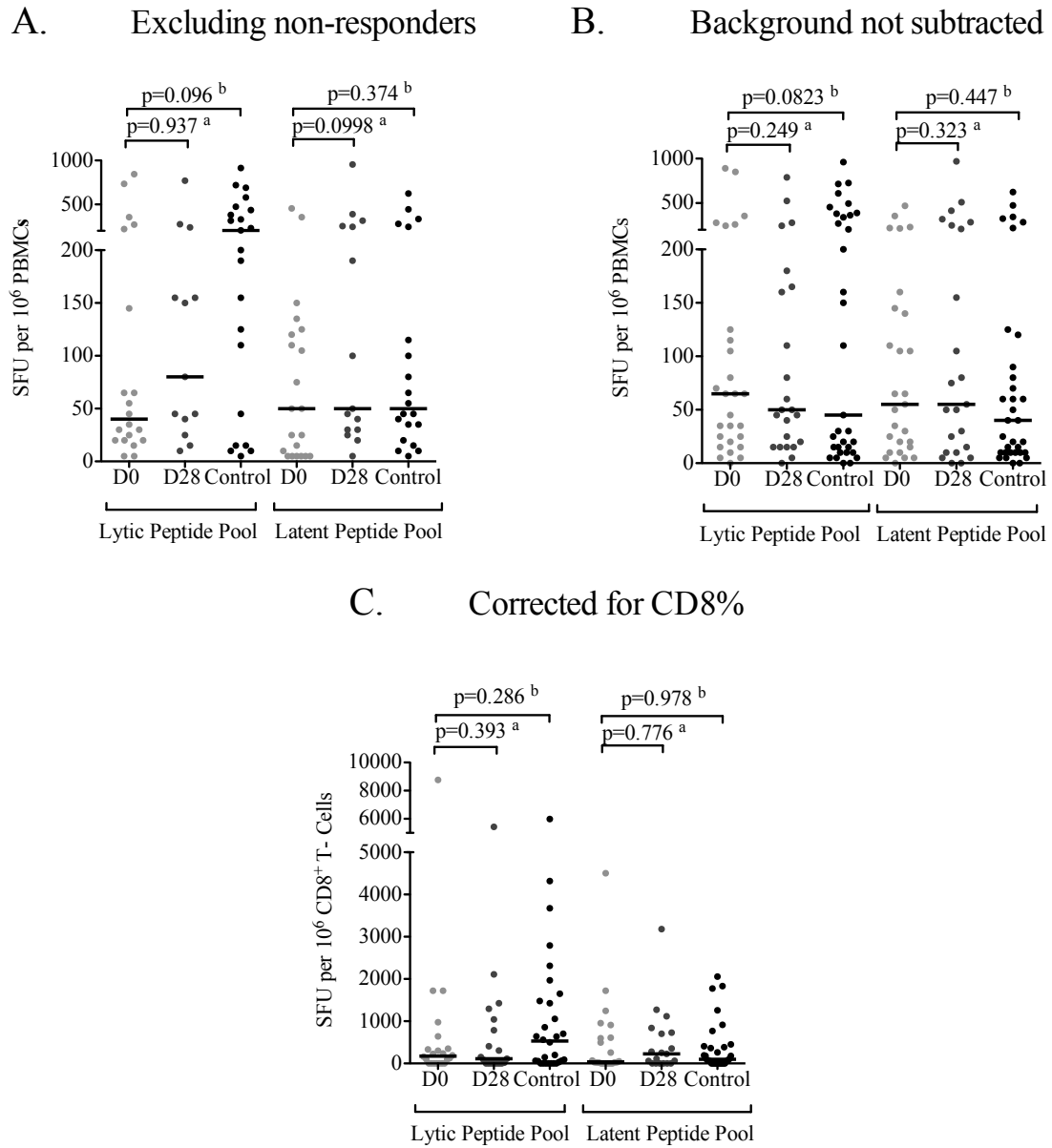


^a *P* value calculated using Wilcoxon rank statistical comparison of matched pairs.

^b *P* value calculated using the Mann-Whitney U comparison of unpaired data.

Figure 3-8 *IFN-γ ELISPOT assays during acute uncomplicated *P. falciparum* malaria infection (*n* = 27), on convalescence in the same donors (*n* = 23), and from healthy age- and sex- matched controls (*n* = 32).*

The solid black lines indicate the median responses for each group.



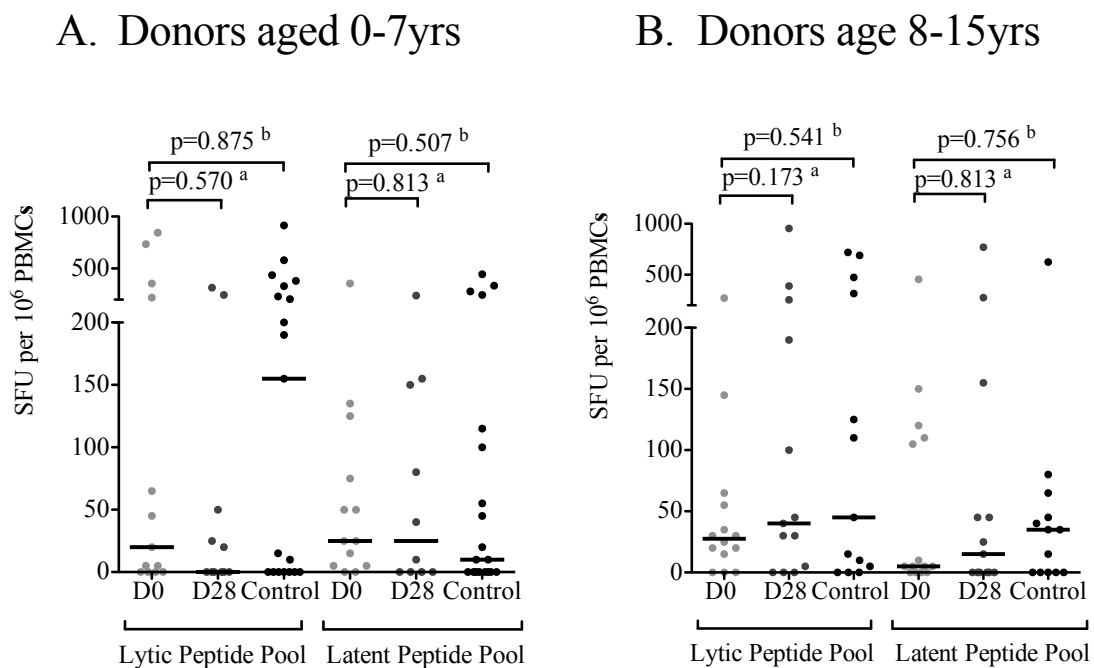
^a P value calculated using Wilcoxon rank statistical comparison of matched pairs.

^b P value calculated using the Mann-Whitney U comparison of unpaired data.

Figure 3-9 $IFN-\gamma$ ELISPOT assays during acute uncomplicated *P. falciparum* malaria infection, on convalescence in the same donors, and from healthy age- and sex- matched controls with **A. non-responders excluded**, **B. without the background subtracted** and **C. corrected for CD8⁺ T-cell percentage**.

The solid black lines indicate the median responses for each group.

Again with the caveat of a small sample size the analysis of these data looking at subgroups of different age ranges (0-7yrs and 8-15yrs) also showed no significant differences in IFN- γ ELISPOT responses between children with acute (Day 0) *P. falciparum* malaria and age- and sex- matched healthy controls. Likewise, the magnitude of IFN- γ ELISPOT responses did not change significantly between D0 and D28 (Figure 3.10).



^a *P* value calculated using Wilcoxon rank statistical comparison of matched pairs.

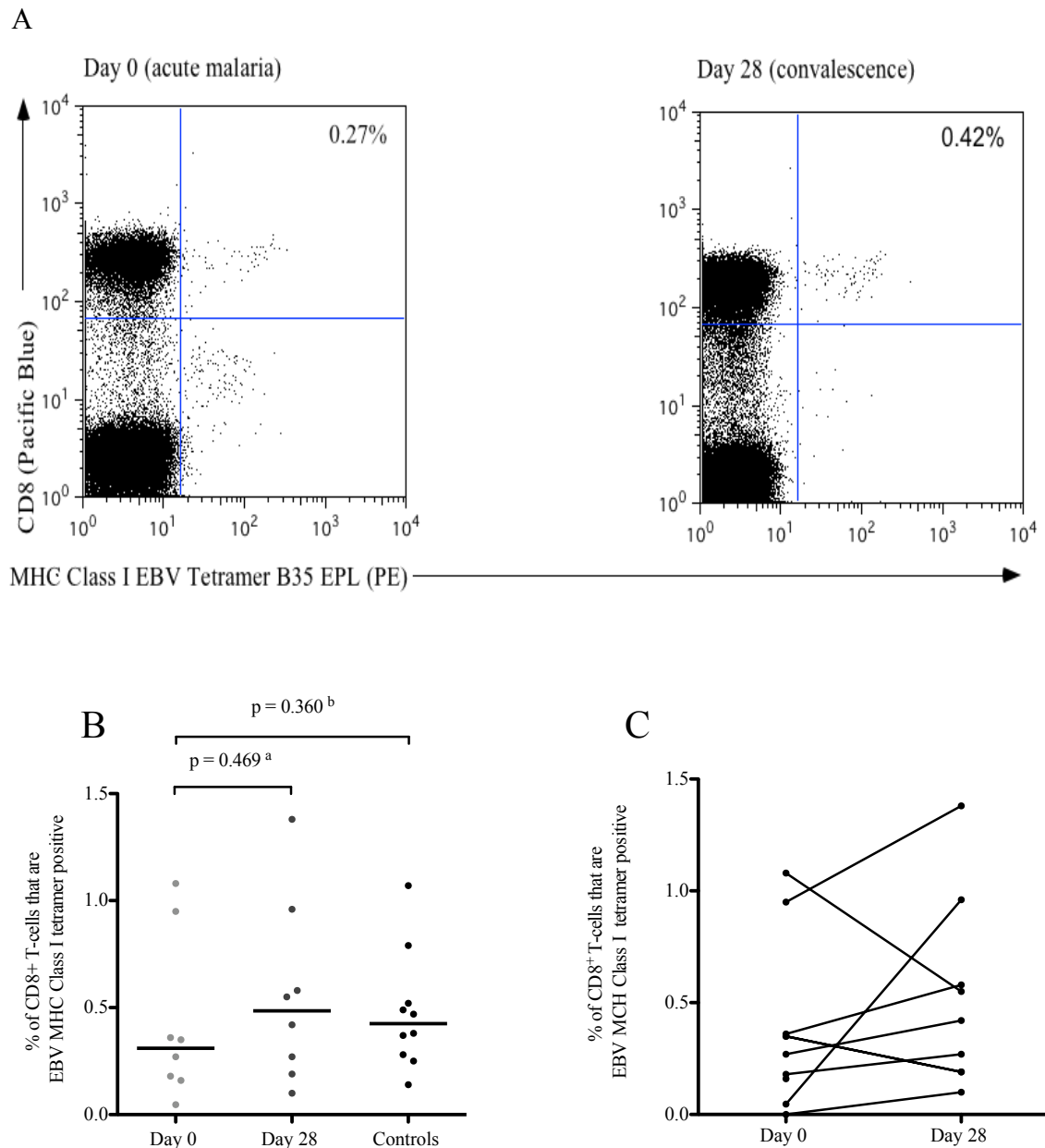
^b *P* value calculated using the Mann-Whitney U comparison of unpaired data.

Figure 3-10 IFN- γ ELISPOT assays during acute uncomplicated *P. falciparum* malaria infection, on convalescence in the same donors, and from healthy age- and sex- matched controls from children aged 0-7 years (A) and 8-15 years. The solid black lines indicate the median responses for each group.

3.3.5 EBV-specific responses, detected by use of MHC Class I Tetramers and regression assays, did not significantly differ between *P. falciparum* infected and uninfected children

Consistent with both the viral load and IFN- γ ELSPOT data there were no significant differences between the percentages of EBV-specific MHC Class I tetramer positive cells during acute (Day 0) infection and age- and sex- matched healthy controls ($p=0.360$). In addition no significant difference was seen in tetramer percentage between Day 0 and Day 28 ($p=0.469$) (Figure 3.11).

To corroborate these findings regression assays were carried out on cryopreserved PBMCs from a subset of acute uncomplicated and severe *P. falciparum* infected children ($n=9$) from the following malaria season. Briefly, PBMCs were infected with B95.8 strain of EBV and seeded in doubling dilutions from 4×10^4 to 1.2×10^3 cells per well. In two replicate wells CSA was added and parallel cultures of uninfected PBMCs were set up as controls. For details of the regression assay see Methods Section 2.4. The strength of regression was expressed as the initial cell seeding necessary to give a 50% incidence of regression among replicate wells as calculated using the Reed-Muench formula. Consistent with our current EBV genome load, IFN- γ ELISPOT analysis and MHC Class I Tetramer data, but in contrast to the original findings by Whittle et al. (Whittle et al., 1984) we saw no significant loss ($p=0.5955$) of regression during acute (Day 0) *P. falciparum* malaria. These assays were conducted on cryopreserved PBMCs but the magnitude of regressive capacity in acute (D0) *P. falciparum* infected children did not significantly differ from those detected using *ex vivo* assays on healthy control children ($p=0.2115$, Figure 3.12).



^a *P* value calculated using Wilcoxon rank statistical comparison of matched pairs.

^b *P* value calculated using the Mann-Whitney U comparison of unpaired data.

Figure 3-11 EBV-specific Tetramer analysis of PBMC from acute *P. falciparum* malaria infected, convalescent and control groups

A. Flow cytometry plots from a representative donor displaying MHC Class I tetramer (B*3501 EPL) against CD8 T-cells during acute *P.falciparum* infection (left) and on convalescence (right), **B.** The percentage of EBV MHC Class I tetramer (B*3501 EPL, A*0201 GLC, A*0201 CLG or B*0801 RAK) positive CD8⁺ T-cells in acute uncomplicated *P.falciparum* infection ($n=8$), on convalescence in the same donors and in age- and sex-matched health controls ($n=10$). **C.** The kinetics of paired MHC Class I tetramer responses, with individual donors at acute infection (Day 0) and on convalescence (Day 28).

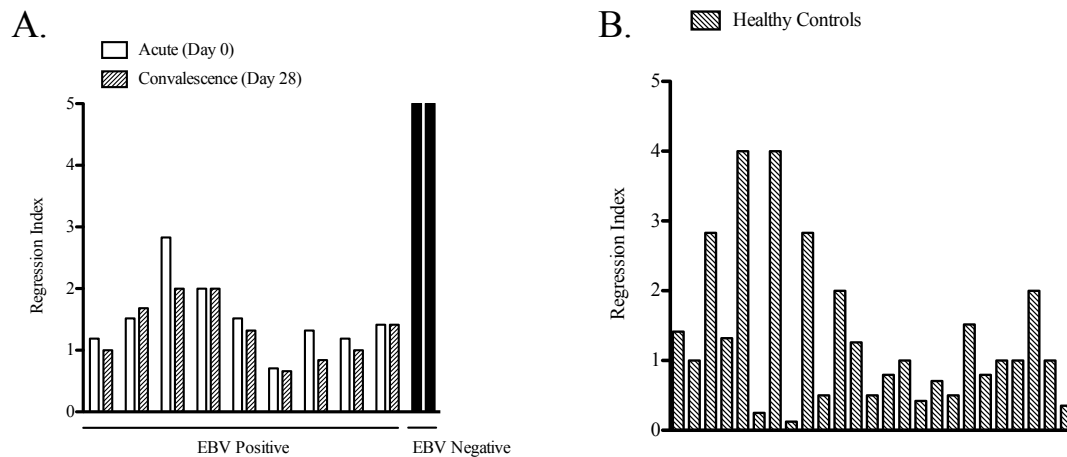


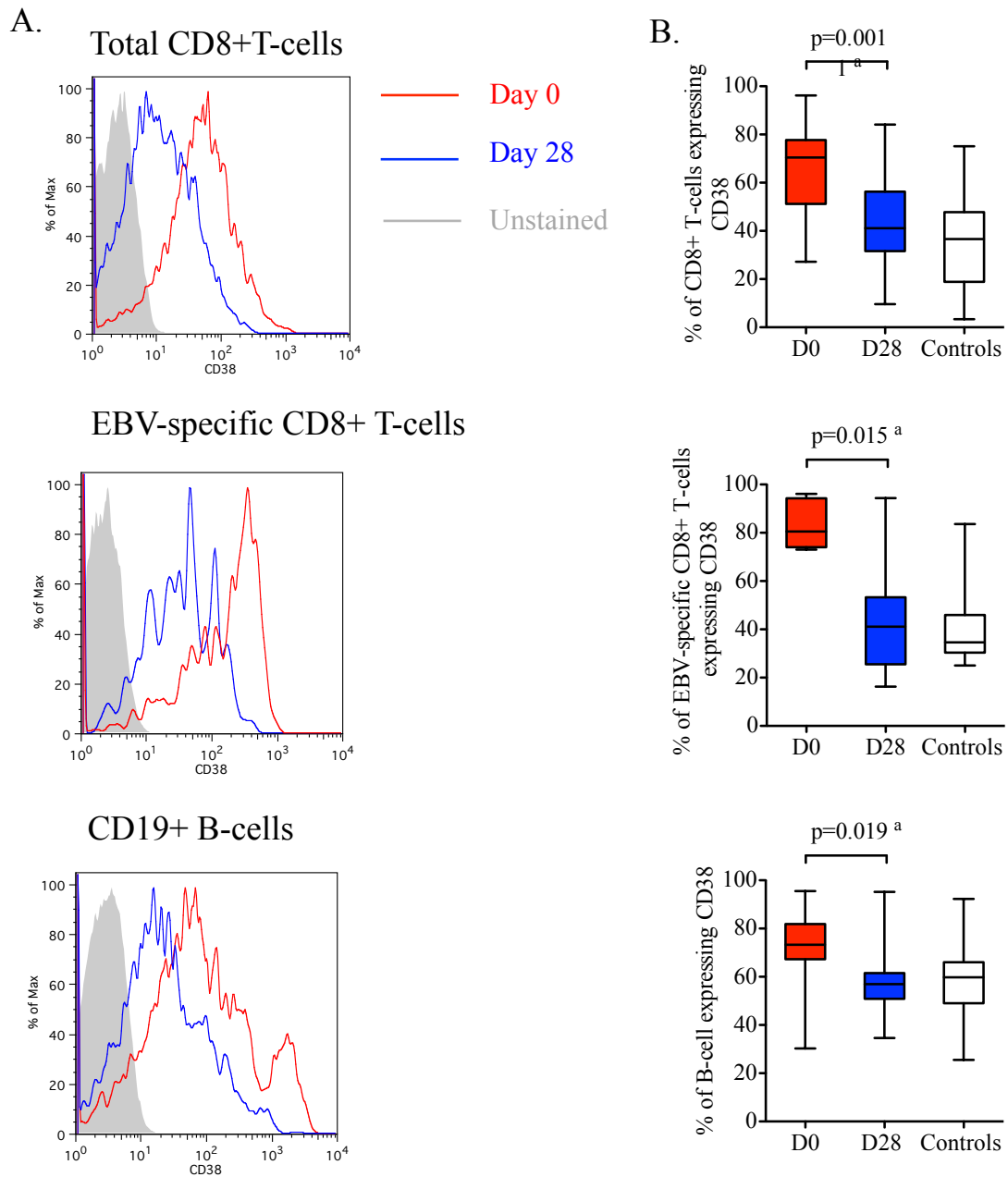
Figure 3-12 Regression assays performed on PBMCs.

*A. Cryopreserved cells collected during acute (open bars) and convalescent phases (patterned bars) of uncomplicated and complicated *P. falciparum* malaria infection. The last two donors are seronegative controls (black bars). B. Ex vivo regression assays performed on freshly isolated PBMCs from healthy control children.*

3.3.6 Immune activation in *P. falciparum* infected and uninfected children

As no differences were seen in T-cell effector responses, next the cell surface phenotype of the EBV-specific T-cells was examined for evidence of activation marker expression, in this case CD38, in the different donor groups. Furthermore, as polyclonal B-cell activation and hypergammaglobulinaemia are recognised as prominent features of malaria (Donati et al., 2004, Whittle et al., 1990) CD38 expression on B-cells was also examined in the different donor groups, a marker of functional maturity on B-cells.

A significantly greater percentage of total CD8⁺ T-cells ($p=0.001$), EBV-specific CD8⁺ T-cells ($p=0.015$), and CD19⁺ B-cells ($p=0.019$) expressed CD38 at the time of presentation with acute *P. falciparum* malaria (Day 0) compared to samples taken at convalescence (Day 28) (Figure 3.13). A similar albeit non-significant trend was seen in CD38 expression on CD4⁺ T-cells ($p=0.068$) (data not shown).



^a *P* value calculated using Wilcoxon rank statistical comparison of matched pairs.

Figure 3-13 *CD38 expression is up-regulated on CD8+, EBV-specific MHC Class I tetramers and CD19+ cells during acute *P. falciparum* infection.*

*A. Representative examples demonstrating increased CD38 APC MFI on CD8+ T-cells (top row), EBV-specific MHC Class I tetramers (middle row) and CD19+ B-cells (bottom row) on Day 0 (red), Day 28 (blue) and on unstained PBMCs (solid grey). B. The percentage of CD38 expressing CD8+, EBV-specific MHC Class I tetramer and CD19+ cells in acute uncomplicated *P. falciparum* malaria infection (red box), on convalescence in the same donors (blue box) and in age- and sex- matched healthy Controls (white box).*

3.3.7 Programmed Death-1 (PD-1) expression in *P. falciparum* infected and uninfected children

During chronic viral infections the PD-1/ PD-L pathway has been shown to play a major role in regulating T-cell exhaustion, a state of T-cell dysfunction defined by poor effector function transcriptionally distinct from functional effector or memory T-cells (Wherry, 2011, Blackburn et al., 2010). Cells expressing this exhausted phenotype are thought to prevent optimal control of infections and tumours. Acute malaria has been shown to have an immunosuppressive effect (Enwere et al., 1999, Kalmbach et al., 2009, Riley et al., 1989a, Riley et al., 1989b), with increased incidence of specific tumours such as eBL in populations experiencing holoendemic malaria exposure (Rochford et al., 2005). The phenotype of the cells in these study groups was further examined to determine whether PD-1 expression altered during acute *P. falciparum* malaria infection. Both cellular and humoral immunity are thought to play important roles in the immune response to malaria (Beeson et al., 2008) therefore surface expression of PD-1 was looked at on both T- and B-cells in the different donor groups.

During acute *P. falciparum* infection the percentage of PD-1 expressing CD19+ B-cells ($p=0.04$) and CD4+ T-cells ($p=0.0085$) was significantly higher than in convalescent samples (Figure 3.14). This trend remains when results are expressed based on absolute numbers of CD19+ B-cells or CD4+ T-cells (Figure 3.14). During acute *P. falciparum* malaria infection numbers of PD-1 expressing CD19+ B-cells ($p<0.0001$) and CD4+ T-cells ($p<0.001$) were significantly higher than in healthy controls and during convalescence this number remained significantly higher than seen in the controls. A similar albeit non-significant trend was seen in PD-1 expression on CD8+ T-cells ($p=0.064$) (data not shown).

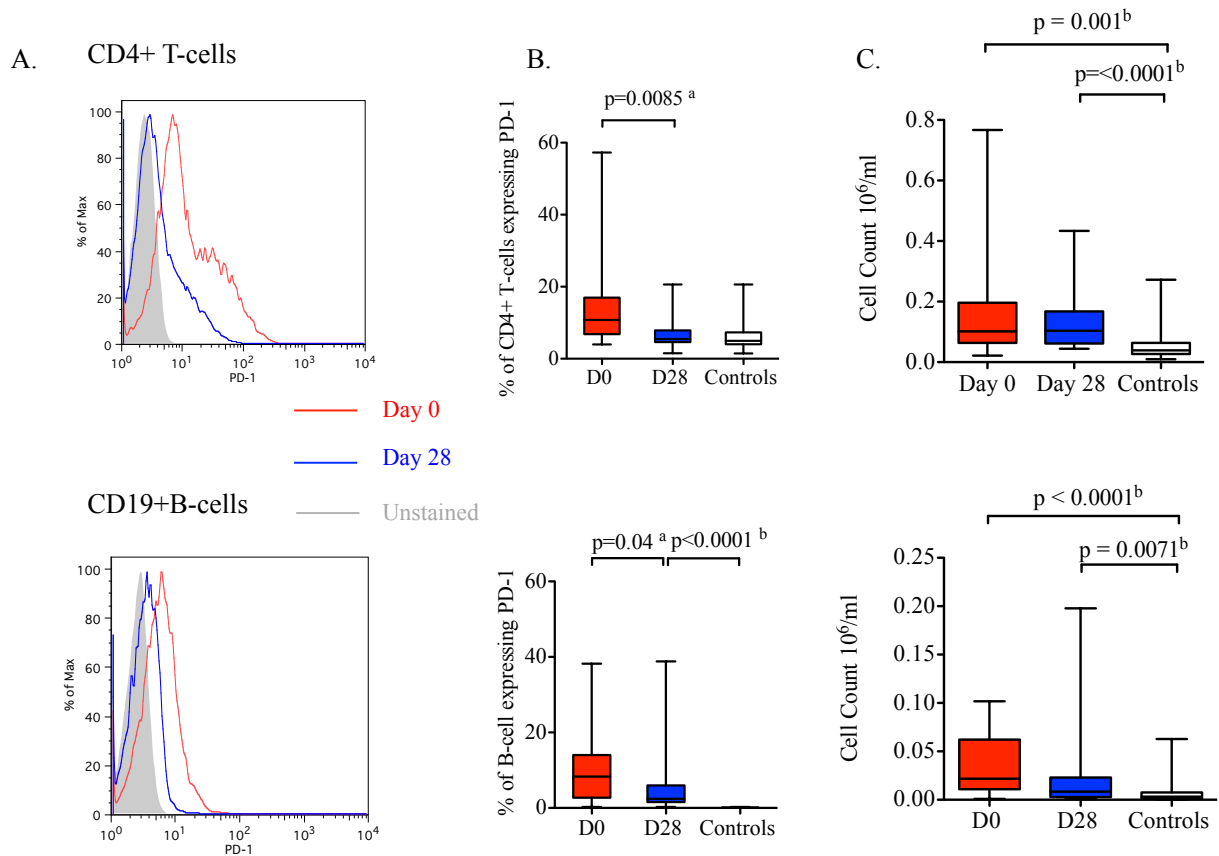


Figure 3-14 Programmed Death-1 (PD-1) expression is up-regulated on CD4⁺ and CD19⁺ cells during acute *P. falciparum* infection in children.

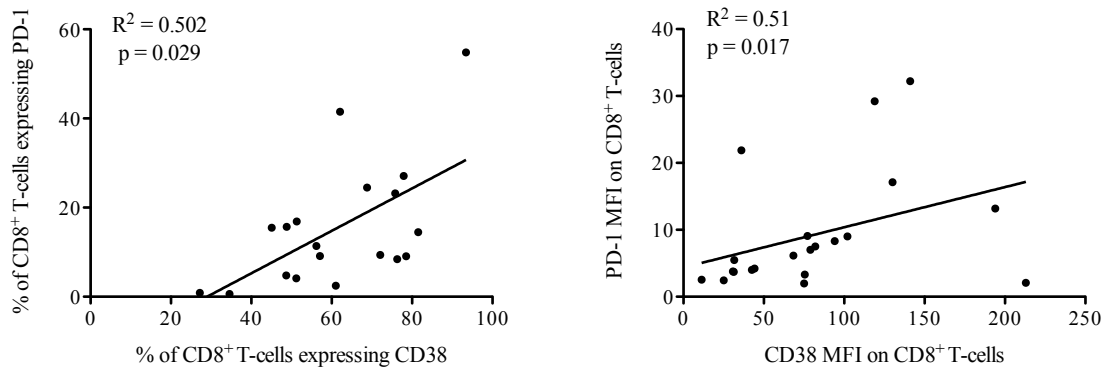
A. Flow cytometry plots demonstrating increased expression of PD-1 on CD4⁺ T-cells (top row) and CD19⁺ cells (bottom row) on Day 0 (red) compared to Day 28 (blue) and on unstained PBMCs (solid grey). B. The percentage of PD-1 expressing, CD4⁺ and CD19⁺ cells in acute uncomplicated *P. falciparum* malaria infection (red box), on convalescence in the same donors (blue box) and in age- and sex- matched healthy controls (white box). C. The absolute number of PD-1 expressing, CD4⁺ and CD19⁺ cells in acute uncomplicated *P. falciparum* malaria infection (red box), on convalescence in the same donors (blue box) and in age- and sex- matched healthy controls (white box). All gates were set using appropriate isotype controls.

^a *P* value calculated using Wilcoxon rank statistical comparison of matched pairs.

^b *P* value calculated using the Mann-Whitney U comparison of unpaired data.

PD-1 has however also been shown to be a marker of activation and differentiation (Sauce et al., 2007). Therefore I looked to see if PD-1 expression correlated with CD38 expression on T- and B-cells. I found that during acute disease (D0) both the percentage and MFI of CD38 expression significantly correlated with PD-1 expression on CD8⁺ T-cells (Spearman's correlation coefficient $R^2=0.502$, $p=0.029$ and $R^2=0.51$, $p=0.017$ respectively, Figure 3.15) and also on CD4⁺ T-cells (Spearman's correlations coefficient $R^2=0.488$, $p=0.040$ and $R^2=0.66$, $p=0.003$ respectively). This supports the hypothesis that PD-1 expression is associated with cellular activation and is not purely a marker of functional exhaustion in these cells. However, this was not found to be the case for CD19⁺ cells (Spearman's correlation $R^2=0.4298$, and $p=0.0663$), data not shown.

A. Total CD8⁺ T-cells



B. Total CD4⁺ T-cells

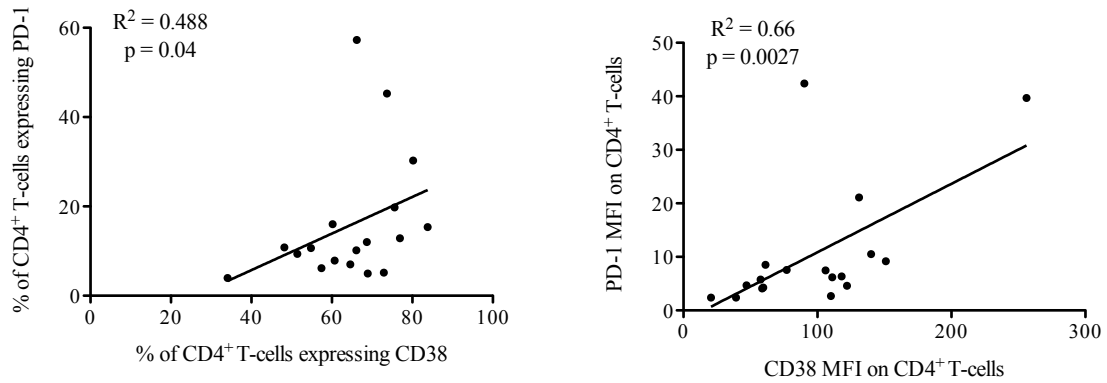


Figure 3-15 *PD-1 expression correlates with CD38 expression during acute (Day 0) uncomplicated *P. falciparum* infection.*

*PD-1 expression is shown on the y-axis and CD38 expression on the x-axis, each dot represents a single donor during acute (Day 0) uncomplicated *P. falciparum* malaria. There is a significant correlation between the percentage of CD38 and PD-1 expression (left) as well as the MFI for CD38 and PD-1 (right) on A. CD8⁺ T-cells and B. CD4⁺ T-cells.*

3.4 Discussion

This work directly assesses the effect of acute *P. falciparum* infection on the number and function of EBV-specific CD8⁺ T-cells *ex vivo*, in parallel with viral genome loads and traditional cell culture-based techniques measuring T-cell mediated regression of outgrowth of autologous EBV transformed B-cells. This study demonstrates that the effect of acute *P. falciparum* infection on cellular immune responses to EBV in The Gambia differs from earlier reports (Njie et al., 2009, Whittle et al., 1984). Now, children with acute uncomplicated *P.falciparum* malaria experience no elevation in EBV genome load in PBMCs compared to age- and sex-matched healthy controls, nor do they have any evidence of reduced numbers or functional impairment of their EBV-specific CD8⁺ T-cells. These different findings may be due to a recent decline in chronic exposure to malaria in infancy and early childhood in The Gambia (Ceesay et al., 2008, Ceesay et al., 2010).

Primary EBV infection, like CMV infection occurs in early childhood in The Gambia (Miles et al., 2007) and more recent data indicates that the age of EBV seroconversion continues to be low with 87% of children between 14 and 18mths of age in The Gambia being EBV seropositive in 2010 (S. Jayasooriya, unpublished data). This is in contrast to recent data from Balfour *et al.* who found that only 63% of college entrants screened in the USA were EBV seropositive leaving 37% still remaining EBV naïve (Balfour et al., 2013).

Loss of regression of EBV-infected B-cell outgrowth during acute malaria was first demonstrated in Gambian children over 25 years ago (Whittle et al., 1984), at a time when the entomological inoculation rate in the country was several fold higher than the current day (Finney et al., 2009, Thomson et al., 1994). Furthermore, work conducted in 2003, Njie *et al.*

detected high EBV loads from PBMCs from Gambian children in the throes of acute malaria. Interestingly these did not fall in convalescent samples taken six weeks later but remained significantly higher than those detected in a cohort of healthy control children, albeit not matched for age- or sex in that report. In the present study, the EBV genome loads (using the same assay as Njie *et al.*) from malaria cases, both in acute and convalescent samples, are lower and not significantly different from those of age- and sex- matched controls, although they remain significantly higher than the range typically seen for healthy virus carriers in the UK population (Njie *et al.*, 2009). Notably, there has been a considerable fall in malaria exposure in The Gambia from 2003 onwards (Ceesay *et al.*, 2008, Ceesay *et al.*, 2010). The likely difference in malaria exposure between the cohorts used by Whittle *et al.* and Njie *et al.* and the current study is highlighted by the dramatic change in entomological inoculation rates from Brefet, a rural community where part of the current study was based: 3.21 in 1991 to 0.62 in 2006 (Finney *et al.*, 2009, Thomson *et al.*, 1994). It therefore seems possible that at the time of these earlier studies the life-long history of *P. falciparum* infection in childhood was still sufficiently chronic/recurrent as to render the EBV-host virus balance susceptible to modulation during a renewed bout of acute malaria. By contrast, given the recent decline in malaria incidence and increasing peak age of *P. falciparum* related hospital admissions in The Gambia (Ceesay *et al.*, 2008, Ceesay *et al.*, 2010), it is plausible that most of our cases were experiencing a primary *P. falciparum* infection. Taken together, this suggests that a single, primary *P. falciparum* infection is not sufficient to impair immunological control of EBV infection.

The work by Moormann *et al.*, who studied two cohorts with differential malaria exposure in the Kisumu and Nandi areas of Western Kenya, is relevant here. They noted higher EBV

genome loads in children, one to four years of age, living in areas holoendemic for malaria, compared to those only sporadically exposed (Moormann et al., 2005). This is consistent with our data and supports the idea that chronic repeated exposure to malaria throughout a child's early years is required to alter the EBV-host balance, leading to high EBV loads and a greater likelihood of further increases linked to acute malarial episodes. To investigate this further would require following up children living in a holoendemic area from birth, to capture their first and subsequent malaria episodes, and to longitudinally examine EBV-specific immune responses and EBV viral loads.

When functionality of EBV-specific CD8⁺ T-cells was measured by IFN- γ ELISPOT, no significant differences between malaria cases, in acute stage or convalescence, and age- and sex- matched healthy controls were observed. Moormann et al. studying Kenyan children found that fewer children between five and nine years of age responded to pools of EBV peptides when coming from an area of high rather than sporadic malaria transmission. They have also suggested that the loss of responses is to EBV lytic but not latent antigens (Moormann et al., 2007, Snider et al., 2012). More recently they have demonstrated no differences in EBV-specific CD8⁺ T-cell frequencies between both cohorts of children, but children living in regions with holoendemic malaria exposure had more differentiated EBV-specific CD8⁺ T-cell populations than those in sporadically exposed areas (Chattopadhyay et al., 2013). Importantly this phenotypic variation was not detected in CMV-specific CD8⁺ T-cells nor in the total CD8⁺ T-cell population. The fact that bed net programmes target 0-5 year old children and that malaria prevalence in The Gambia peaks in children above this age (16 years) make it conceivable that the most pronounced difference is detected in children above this age group (Satoguina et al., 2009). Of additional interest, despite a preservation of EBV-

specific CD8⁺ T-cell responses, we find that PD-1, a marker reported to signify cellular exhaustion, is significantly up-regulated in acute malaria and does not decline to control levels by day 28. However, PD-1 expression significantly correlates with CD38 expression on CD4⁺ and CD8⁺ T-cells, which supports recent findings in HIV that PD-1 is not purely a marker of cell exhaustion (Sauce et al., 2007) and reflects recent activation.

The work in this Chapter has limitations that are worth discussion. Although previously reported data suggest a dramatic decline in malaria exposure in the country as a whole, the study design precludes exact knowledge of the exposure history of the study participants. However, of note 95% of three year old children followed up longitudinally in a birth cohort adjacent to our study's catchment area had no serological evidence of malaria exposure (Ceesay et al., 2008), and a cohort study conducted in Brefet in the 2009 transmission season only identified one clinical case of malaria (Nogaro et al., 2011). In addition to this we found that only 24% of control children, mean age 7 years (range 1.5-16 years), were MSP-1(19) seropositive (Figure 3.2). This is lower than previously quoted figures (30%, 0-7 years and 46%, 8-10 years) (Corran et al., 2004). Recent publications from The Gambia from differing peri-urban sites also confirm this decline (Satoguina et al., 2009), with one study demonstrating only a 4% seropositivity to MSP-1(19) in 0 to 14 years olds in 2009 (Ceesay et al., 2010).

Previous case-control studies have demonstrated that acute malaria infection can alter EBV host balance leading to an increase in EBV genome loads (Donati et al., 2006, Rasti et al., 2005, Yone et al., 2006). The cross-sectional cohort studies conducted by Moormann et al. indicate that chronic malaria infection also plays a role in tipping the balance in favour of the

virus (Moormann et al., 2005, Moormann et al., 2007, Snider et al., 2012). Our findings suggest that there is no impairment of EBV immunity during an acute *P. falciparum* malaria infection and therefore suggest that alteration in EBV host balance is likely to be dependent on cumulative prior infections. A further longitudinal study of primary and cumulative acute *P. falciparum* infections and their impact on EBV host balance would be required to test this hypothesis.

Our findings, however, have more general implications adding to the mounting evidence that the decline in malaria seen in some parts of sub-Saharan Africa have wider implications than a reduction in morbidity and mortality from malaria alone. Additional changes have been seen in the rates of invasive bacterial diseases such as non-typhoidal salmonella in The Gambia (Mackenzie et al., 2010, Obaro and Greenwood, 2011), and all cause mortality in children within regions where a reduction in malaria has been demonstrated (Snow and Marsh, 2010). Data to establish whether our observation translates into a reduced incidence of eBL in The Gambia are needed.

CHAPTER 4 : PHENOTYPIC AND FUNCTIONAL CONSEQUENCES OF EPSTEIN-BARR VIRUS INFECTION IN INFANCY

4.1 Introduction

EBV is a ubiquitous gamma herpesvirus associated with occasional severe primary infections, several malignancies and significant pathology in immunosuppressed hosts, but it does not cause significant morbidity in the majority of those infected. In The Gambia most people are infected in infancy in contrast to most developed countries where the majority of primary infections occur at a much later stage, often in adolescence (Biggar et al., 1978a, Hislop et al., 2007b). Paradoxically it is estimated that about a quarter of those infected in adolescence will develop a sometimes-severe disease, AIM, whereas those infected in infancy tend to have minor self-limiting illnesses that often go undetected. Persistent EBV infection is associated with malignancies, BL, NPC and a subset of Hodgkin lymphomas. The former is found to be endemic in the malaria belt in Africa peaking in children 5-7years of age. A likely factor contributing to the aetiology of eBL is early primary EBV infection alongside high levels of malaria exposure (Rochford et al., 2005). In addition the low age of EBV seroconversion is thought to play a significant role in increasing the risk of developing subsequent B-cell malignancies (Chabay and Preciado, 2012).

In contrast to a paucity of studies of EBV infections in infancy and childhood, many studies in adults have characterised cellular immune responses, both to CD8+ and to a lesser extent CD4+ T-cells, *in vivo* during primary infections (AIM) (Amyes et al., 2003, Callan et al., 1996, Callan et al., 1998b, Catalina et al., 2001, Faint et al., 2001, Hislop et al., 2002, Hislop et al., 2001, Hislop et al., 2005, Hoshino et al., 1999, Long et al., 2005, Long et al., 2011,

Mautner and Bornkamm, 2012, Pudney et al., 2005, Steven et al., 1997, Steven et al., 1996, Woodberry et al., 2005). There are unusually large numbers of CD8+ T-cells during AIM (Callan et al., 1998b). Lytic epitope responses seen in AIM can account for 1-40% of the total CD8+ T-Cell population. Responses to IE and E proteins of the lytic cycle usually dominate. The latent cycle protein responses tend to be smaller accounting for only 0.1-5% of the CD8+ T-Cell population. These usually focus on the family of EBNA proteins, 3A, 3B and 3C. These CD8+ T-cells have been well described in adults and display a phenotype consistent with recent antigen stimulation. They are perforin positive with direct *ex vivo* cytotoxic function (Callan et al., 2000, Steven et al., 1997, Steven et al., 1996), express activation marker CD38 and cell cycling maker Ki-67 (Callan et al., 2000, Catalina et al., 2001, Hislop et al., 2002, Hislop et al., 2005). These cells die rapidly *in vitro* unless given an antigen stimulus, expressing high levels of pro-apoptotic protein Bax and low levels of the anti-apoptotic bcl-2 (Callan et al., 2000, Dunne et al., 2002, Tamaru et al., 1993). Consistent with this, CD8+ T-cells are rapidly culled in the weeks post infection, lytic responses more heavily than latent. It is therefore not surprising that quantitative changes are seen in the dominance of lytic and latent epitope responses with the picture seen during persistent infection often varying greatly from that of AIM (Hislop et al., 2002, Woodberry et al., 2005). The CD4+ T-cell pool is not greatly expanded in AIM, but as with the greater CD8+ T-cell responses, there is a rapid peak and then decline of EBV-specific CD4+ T-cells within weeks (Callan, 2003, Hislop et al., 2007b, Precopio et al., 2003). EBV-specific CD4+ T-cells have been less well characterised in view of the relative lack of well-defined epitopes and the difficulties surrounding HLA class II tetramer manufacture.

Immune responses in adult healthy carriers with persistent infection and no history of AIM have been described (Amyes et al., 2003, Hislop et al., 2001, Hislop et al., 2007b, Tan et al., 1999). Asymptomatic virus carriers maintain T-cell memory both to latent and lytic cycle antigens. With respect to latent antigen responses, CD8+ T-cells specific for known EBV epitopes are relatively common (ranging from 0.05-1% of CD8+ T-cells in the blood) and are typically ten-fold more numerous than their CD4+ counterparts. CD8+ T-cells specific for lytic cycle antigens are generally even higher (0.2-2%), while the CD4+ lytic antigen response is less well studied but again appears to be less numerous than the CD8+ response. (Hislop et al., 2007b, Leen et al., 2001, Long et al., 2005) The CD8+ memory T-cells tend to display a resting phenotype, lacking activation markers and expressing much higher levels of bcl-2 than AIM T-cells. There are however some which still express perforin and are cytotoxic. (Callan et al., 1998b, Catalina et al., 2002, Dunne et al., 2002, Faint et al., 2001, Hislop et al., 2001)

There is a paucity of data in the literature looking at asymptomatic primary infections largely due to the difficulty in identifying donors without any clinical symptoms undergoing a primary EBV infection. Four patients undergoing silent seroconversion were incidentally identified within a vaccine trial and found not to have the massive T-cell expansions seen in AIM, but in contrast maintained homeostatic T-cell control, despite having high EBV genome loads in the blood equivalent to those seen in AIM. There is less known about the immunological and virological response to EBV infection when it occurs in infancy except that it has been shown to occur without significant lymphocytosis and with reduced and delayed viral antibody responses (Fleisher et al., 1979). One previous study conducted by a group at the University of Massachusetts looked at responses in nine children indirectly

assessing cytotoxic T lymphocyte (CTL) responses using recombinant vaccinia constructs expressing single latent genes only (Tamaki et al., 1995). With the caveat of the sample size, they demonstrated that all children had CTL responses against one or more of the EBNA proteins 3A, 3B and 3C, similar to the latent responses reported for adults.

The impact that early EBV infection might subsequently have on immune function has again been little explored. Recent work by Miles *et al.* on infants in The Gambia looked at CMV infection, another member of the herpes virus family, demonstrating a profound effect on the CD8⁺ T-cell compartment with an increase in the proportion and absolute number of differentiated CD8⁺ T-cells (Miles et al., 2008b, Miles et al., 2008c). The CD4⁺ T-cell compartment was also affected but less dramatically with only a small increase in cells expressing a highly differentiated phenotype, and little change in the total CD4⁺ T-cell frequencies (Miles et al., 2008a). Whether EBV has any effect on the CD8⁺ T-cell compartment and how this might interact with what is known about early CMV infection is not known.

This chapter represents a detailed study of EBV-specific T-cell responses from a cohort of 120 children followed longitudinally and describes cellular immune responses in Gambian children having undergone recent primary EBV infections and also captures six children undergoing silent seroconversion. It provides a comprehensive analysis of EBV-specific CD8⁺ T-cells in these children, including a description of the immunodominant responses seen to a broad range of lytic and latent EBV antigens, viral genome loads and detailed *ex vivo* phenotypic analysis of both total and EBV-specific CD8⁺ T-cells.

4.2 Methods

4.2.1 Study Design and Donors

Gambian children (n=120), aged between 14 and 18 months, were initially recruited into the study cohort from the MRC Sukuta field site when they were attending for their routine DTP vaccination booster. They were invited to participate by field-workers who obtained informed consent from their mothers in their local languages. Ethical approval was obtained from the Scientific Coordinating committee, MRC Gambia, and local Gambian government ethics committee. Children underwent a clinical assessment prior to enrolment into the study and were excluded if their weight was below the normal expected range for their age as indicated on their Infant Welfare Growth Chart or had abnormal findings, such as a fever or signs and symptoms were detected during clinical assessment. Children were followed up at weekly intervals for the first three visits, with a fourth visit conducted six months after the third (Figure 4.1). Study recruitment continued from June 2010 until April 2011.

Study participants were allocated ID numbers and a card detailing this number enabling us to identify them and allowing them to access the clinical services provided by the MRC at the Sukuta field site for the duration of the study. Chapter 2, sections 2.1 and 2.2 details samples taken and processing.

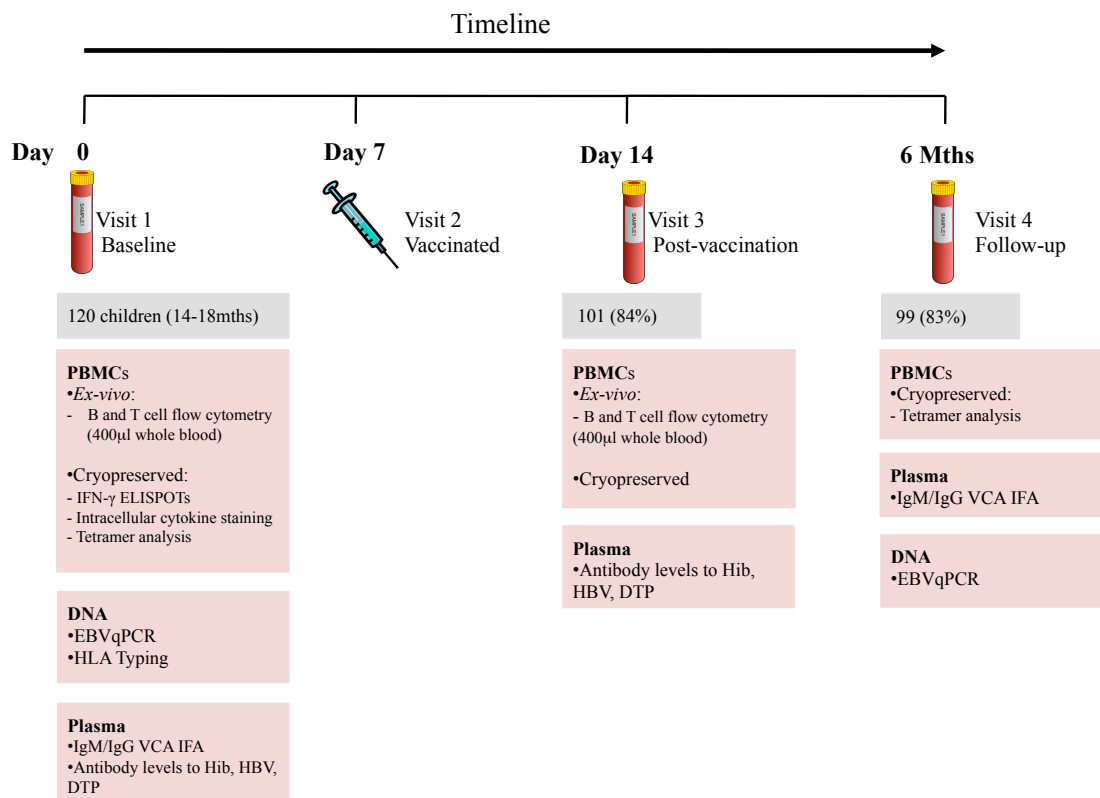


Figure 4-1 *Study timeline.*

4.2.2 Rapid Malaria Test (RMT)

When clinically indicated, children were tested with rapid malaria tests (OptiMAL-IT) which detect parasite lactate dehydrogenase (pLDH)). If positive children were then transported to the clinical services at MRC Fajara for further treatment as indicated.

4.2.3 Cellular immunological Assays

Ex-vivo screening IFN-γ ELISPOT responses of thawed PBMCs to 21 peptide pools combining 63 latent and 43 lytic-cycle antigens covering a broad range of HLA types were performed. Laboratory and flow cytometry methods and analysis are detailed in Chapter 2, sections 2.8, 2.13-2.17.

4.3 Results

4.3.1 Demographics and categorisation of study population

As Figure 4.1 illustrates 120 children between the ages of 14 and 18 months were enrolled into the study at baseline. Of these, 99 (83%) children remained in the study until completion at the six-month time point. Table 4.1 illustrates that the children who did not continue to participate in the study did not significantly differ in age, sex, weight, haemoglobin, leucocyte and lymphocytes counts, from those who continued to participate.

Only one child at enrolment screening was found to be malaria parasite positive by rapid testing following their initial clinical assessment prior to consent. They were not enrolled into the study and received appropriate treatment as per current guidelines from the MRC Clinical Services.

Table 4-1 Age, sex, haemoglobin, weight and cell counts in study participants and dropouts.

Baseline Characteristics	Study Participants (n=99)	Drop outs (n=21)	P value ^a
Age (mths)	15 (14 – 17)	15 (14 – 16.50)	0.79
Sex (F: M)	1:1.5	1:2	-
EBV negatives	13 (13%)	7 (33%)	-
Weight (kg)	9.52 (8.90-10.24)	9.28 (8.85 -9.65)	0.14
Haemoglobin (g/dl)	10.10 (9.28-11.00)	9.8 (8.18-11.03)	0.40
Leucocyte Count (x10 ⁹ /L)	10.90 (8.85-12.63)	11.40 (10.35-13.48)	0.15
Lymphocyte (%)	56.85 (49.80-63.33)	61.90 (50.03-65.35)	0.18

Median values are stated and inter-quartile ranges are shown in brackets

^a p value calculated using Mann-Whitney U statistical comparison of unpaired data

In order to determine the EBV serological status of the 120 study participants they were screened using IgG and IgM VCA immunofluorescence assays (Methods section 2.8.1). For simplicity children are referred to in the groups determined by their serological status. There were six children for whom the results were ambiguous, since they were serologically negative but with detectable EBV viral genome loads (median 4500, range 363-12300). It is possible that these could represent very early infections and consistent with this they all became IgM–IgG+ by the visit four-time point, however they could have seroconverted at any time over the six-month follow-up period. Three of these donors had sufficient cell numbers that enabled them to be screened using the IFN- γ ELISPOT assays (Methods section 2.12), and no responses were detected at visit one. As the status of these six children could not be confidently resolved they were conservatively excluded from further analysis reducing the usable number enrolled to 114.

At visit one 71 (62%) children were classified as EBV infected with IgM–IgG+ serological responses (Table 4.2). From previous studies conducted at the Sukuta field site it is known that by nine months of age only 24% of children are EBV infected (David Miles, Personal Communication) one can therefore assume that most had been infected within the last 6-12 months. There were three (2.6%) children that were IgM+IgG –/+ and 40 (35%) children that were IgM–IgG– with confirmatory undetectable viral loads at visit one. At the visit four-time point, of the 40 previously IgM–IgG– visit one donors, 18 (45%) became IgM–IgG+, two (5%) became IgM+IgG+, 13 (33%) remained IgM–IgG– and seven dropped out. The three children that were IgM+IgG –/+ at visit one had become IgM–IgG+ by visit four.

Table 4-2 Serological status of study participants at baseline (visit one) and six months later (visit four).

Serological Status	Visit one (n=114)	Visit four (n=93)
IgM–IgG–	40 (35%) ^a	13 (14%)
IgM+IgG–	2 (2%) ^b	0
IgM+IgG+	1 (1%) ^b	2 (2%)
IgM–IgG+	71 (62%) ^c	78 (84%)

Number of children stated, percentage of total population given in brackets.

Shaded cells indicate children considered EBV infected and no shading those that are EBV negatives

^a Out of the 40 IgM–IgG– children at visit one 13 remained seronegative, two became IgM+IgG+ 18 became IgM–IgG+ and seven dropped out

^b All three IgM+IgG–/+ donors at visit one became IgM–IgG+

^c Out of the 71 IgM–IgG+ donors at visit one 14 dropped out

4.3.2 Size of lymphocyte populations in the blood of IgM–IgG+ children compared to IgM–IgG– children

The overall leucocyte counts did not differ between the IgM–IgG+ and IgM–IgG– children with medians of $11.30 \times 10^9/L$ (range 5.8-21.90) and $10.50 \times 10^9/L$ (range 6.9-17.70) respectively, being of a similar order to the overall counts seen in the total cohort, see Table 4.1. Lymphocyte populations in children found to be IgM–IgG+ at the visit one time point, i.e. those assumed to have acquired EBV within the last six months to a year were examined and compared to the subsets found in IgM–IgG– children. Figure 4.2 demonstrates that the total absolute CD4+, CD8+ and CD19+ cell counts did not significantly differ between IgM–IgG+ and IgM–IgG– Gambian children. For five IgM–IgG– children and one IgM–IgG+ there was no white cell count available. There was one donor with a very high B-cell count and percentage, they were serologically positive with an undetectable viral load and did not have an expansion in their memory B-cell population. Although the median absolute

number of CD19+ CD27+ memory B-cells also did not significantly differ, expansion of this population's cell number was only seen in some IgM–IgG + children.

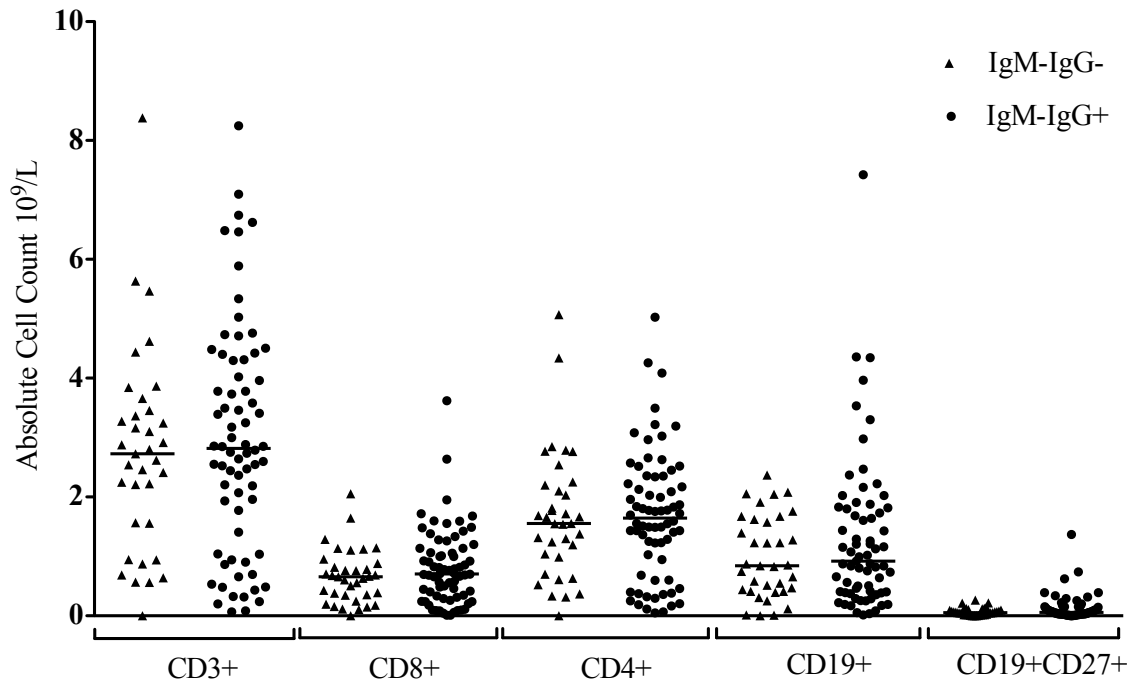


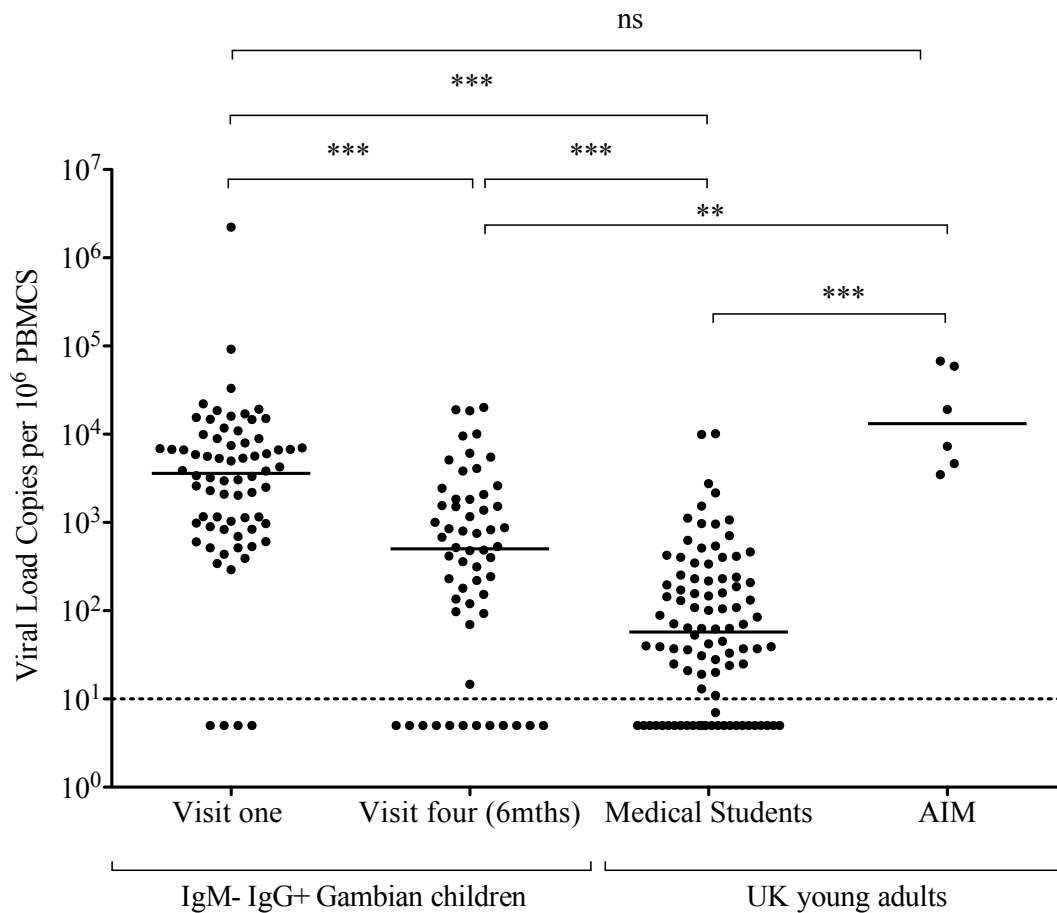
Figure 4-2 Size of Gambian children lymphocyte populations in the blood of IgM–IgG – (n=35) compared to IgM–IgG + children (n=70).

4.3.3 EBV genome loads in PBMCs

The EBV viral genome loads at visit one and six months later at visit four were compared in Gambian children found to be IgM–IgG+. Figure 4.3 demonstrates that there was a spread of values with a median of 3607 copies per 10⁶ PBMCs at visit one, which dropped a significant 7-fold by visit four to a median of 505 copies per 10⁶ PBMCs (p<0.001). The fact that the IgM–IgG+ children had high EBV genome loads at visit one, which subsequently dropped over the following six months, supports the premise that these children are likely to have

undergone recent primary infections within the last six months to a year. The EBV genome loads seen at the six-month time point were equivalent to those previously described in healthy Gambian children by Njie *et al.* albeit from older children, aged three to fourteen years of age (Njie *et al.*, 2009).

These EBV genome loads were compared to donors from the United Kingdom, both in long-term carriers and those with a diagnosis of AIM. EBV genome loads in Gambian children who were IgM–IgG+ were significantly higher than those seen in UK medical students (18-25yrs). The IgM–IgG+ Gambian children for whom EBV genome loads were repeated after a six month time period remained significantly higher than UK medical students (18-25yrs) (UK medical student viral genome loads kindly provided by A. Pachnio performed using the same viral genome assay used in this study at the University of Birmingham Institute for Cancer Studies). The EBV genome loads in IgM–IgG+ Gambian children did not significantly differ from those seen in UK donors with AIM (AIM viral genome loads kindly provided by Dr. A. Hislop performed using the same viral genome assay used in this study at the University of Birmingham Institute for Cancer Studies) (Figure 4.3).



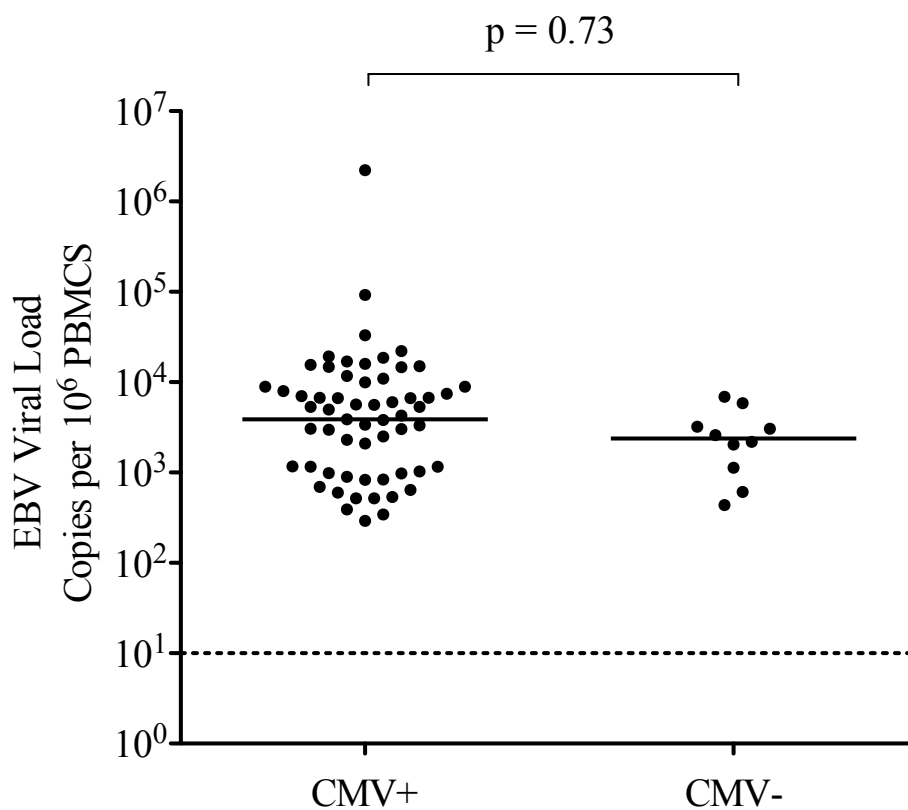
P values calculated using Dunn's Multiple Comparison Test (one way analysis of variance (ANOVA)). ** $P < 0.01$ *** $P < 0.001$

Figure 4-3 *EBV genome loads at visit one ($n = 70$) and at visit four after six months ($n=58$) in IgM–IgG+ Gambian children, adolescent Caucasian medical students ($n=94$), and Caucasian adults with acute infectious mononucleosis (AIM) ($n=6$).*

The dashed line represents the lower limit of detection for EBV genomes in the assay. Donors below the dashed line had undetectable viral loads.

In view of current literature demonstrating the significant impact of early CMV infection on the CD8⁺ T-cell compartment (Miles et al., 2008b, Miles et al., 2008c) the CMV status of all children was determined at visit one. Eighty-four percent of these children (14-18mths) were

CMV seropositive. This is consistent with D. Miles *et al.* data that demonstrated CMV infection in 85% of his infant cohort at 18mths of age in The Gambia (Miles et al., 2008b). EBV viral genome loads in the majority CMV positive versus CMV negative Gambian children were compared, excluding those children classified as IgM–IgG– (n=40). Figure 4.4 shows no significant difference in EBV genome loads between the two CMV groups.

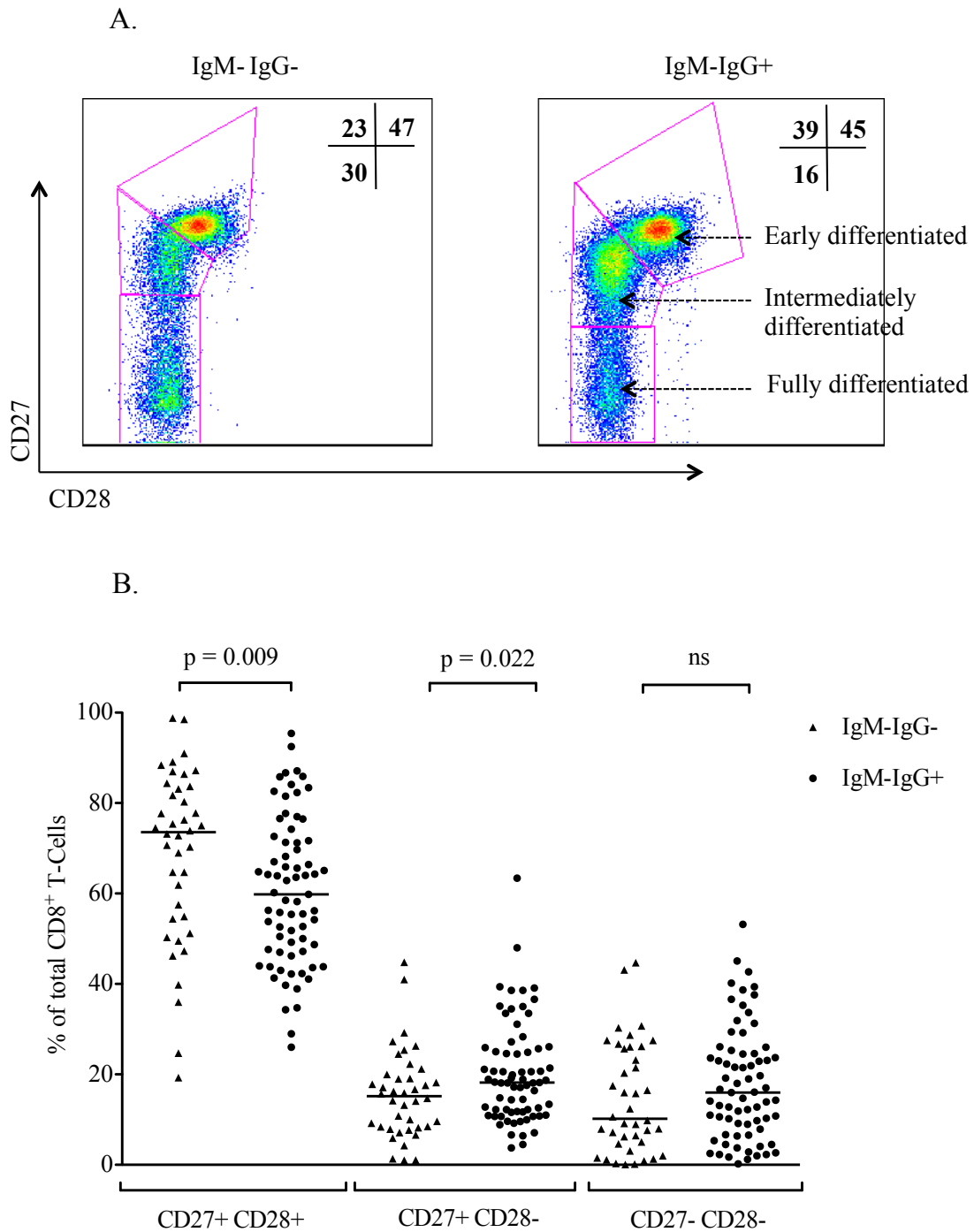


P value calculated using the Mann-Whitney U comparison of unpaired data.

Figure 4-4 *EBV genome loads at visit one according to CMV status.*

4.3.4 CD8+ T-cell subset distributions in IgM–IgG+ children compared to IgM–IgG– children

The phenotype of the total CD8+ T-cells of IgM–IgG+ donors was assessed using *ex vivo* flow cytometry staining at visit one. Cells stained for the co-stimulatory molecules CD27 and CD28 were divided into early (CD27+ CD28+), intermediate (CD27+ CD28–), and fully differentiated (CD27– CD28–) to determine the differentiation status of the total CD8+ T-cell population (Appay et al., 2002a, Hamann et al., 1999, van Lier et al., 2003). Figure 4.5A demonstrates representative flow plots for IgM–IgG– and IgM–IgG+ donors illustrating that the ‘intermediately differentiated’ subset (CD27+ CD28–) of total CD8+ T-cells was significantly larger in the IgM–IgG+ group compared to those who were IgM–IgG– (Figure 4.5B) and as a consequence the early differentiation (CD27+ CD28+) subset was lower in the IgM–IgG+ group.



Percentages of total CD8⁺ population given in top right-hand corner of plots.

Figure 4-5 CD27 and CD28 co-staining on IgM-IgG⁻ and IgM-IgG⁺ children.

A. Representative flow cytometry plots demonstrating CD27 and CD28 co-staining of CD8⁺ T-cells from an IgM-IgG⁻ (left) and IgM-IgG⁺ (right) Gambian child. B. Differentiation status of total CD8⁺ T-cell populations in IgM-IgG⁻ and IgM-IgG⁺ Gambian children displaying percentage of total CD8⁺ T-cells expressing early (CD27⁺CD28⁺), intermediate (CD27⁺CD28⁻), and fully differentiated (CD27⁻CD28⁺) phenotypes.

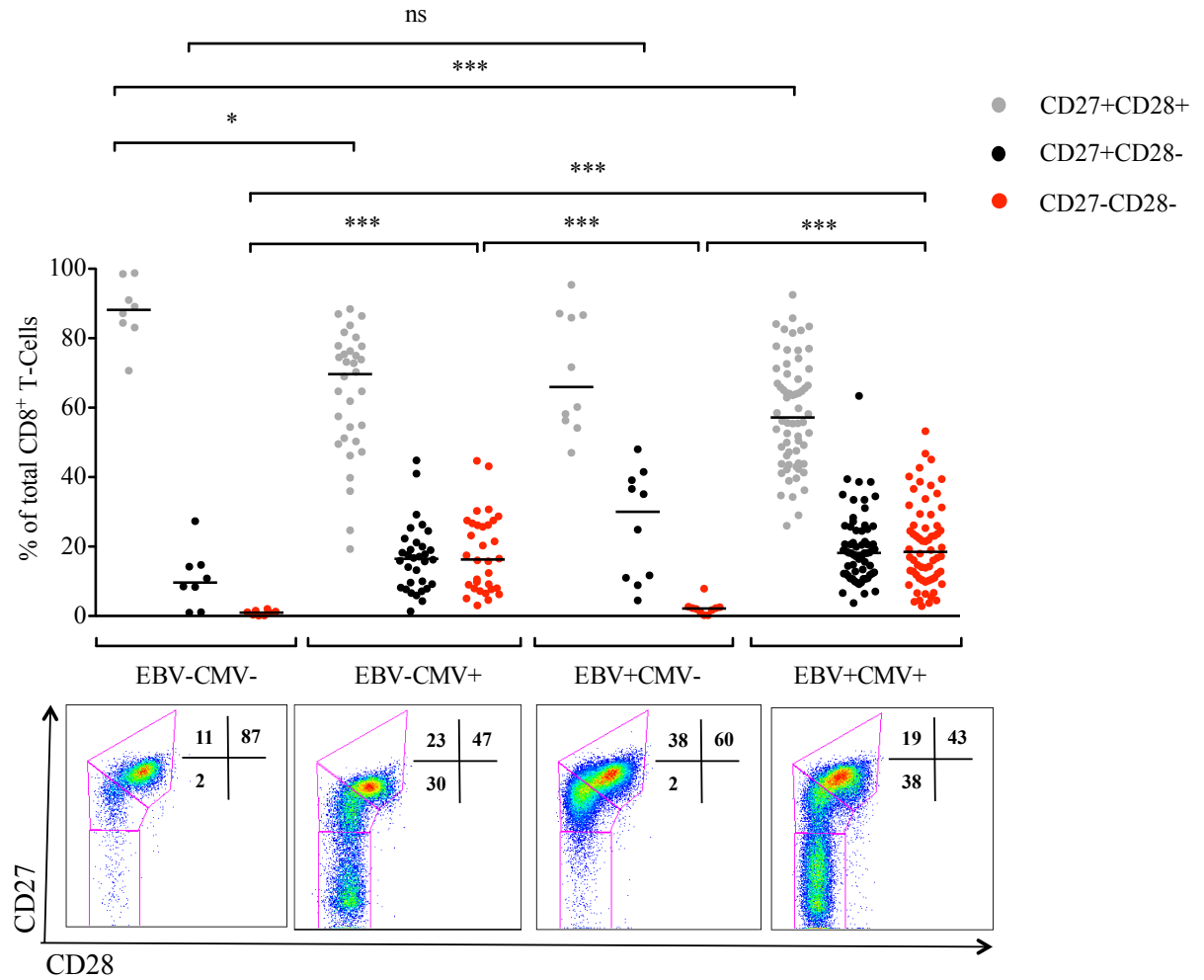
A potential confounder here is CMV infection. Miles *et al.* has shown that CMV infected Gambian infants have more terminally differentiated total CD8+ T-cells than uninfected infants. As previously mentioned CMV status was determined at visit one on all Gambian children in this study. Table 4.3 illustrates both combined CMV and EBV status on all of the children in the study. The majority of children (56%) were found to be dually CMV and EBV infected.

A previous study shows that Gambian children in the study site of Sukuta, tend to be infected earlier with CMV than with EBV, 66% and 18% of children were CMV and EBV infected respectively at nine months of age (Holder *et al.*, 2010). CD8+ T-cell differentiation status was therefore assessed according to EBV and CMV status in the following groups, CMV-EBV-, CMV+EBV-, CMV-EBV+ and CMV+EBV+, to determine the effect of single and co-infection with CMV and EBV on CD8+ T-cell differentiation status (Figure 4.6). Consistent with previous findings (Miles *et al.*, 2007), CMV infection alone significantly reduces the percentage of 'early differentiated' CD8+ T-cells and increases the percentage of fully differentiated CD8+ T-cells. With the caveat of a small sample size, there is a non-significant decline in the percentage of 'early differentiated' CD8+ T-cells and increase in the percentage of 'intermediately differentiated' CD8+ T-cells in the EBV infected CMV negative donors compared to non-infected donors. The differentiation phenotype of co-infected Gambian children's total CD8+ T-cells mirrored that of solitary CMV infection.

Table 4-3 *Two-way table illustrating EBV and CMV status of Gambian children aged 14-18mths at visit one (Day 0).*

	CMV infected	CMV uninfected	Total
EBV infected	64 (56%)	10 (9%)	74 (65%)
EBV uninfected	32 (28%)	8 (7%)	40 (35%)
Total	96 (84%)	18 (16%)	114

Number of children stated, percentage of total population given in brackets.



P values calculated using Dunn's Multiple Comparison Test (one way analysis of variance (ANOVA)). * $p < 0.05$ ** $P < 0.01$ *** $P < 0.001$

Percentages of total CD8⁺ T-cell population given in top right-hand corner of plots.

Figure 4-6 CD8⁺ T-cell differentiation determined by CD27 and CD28 co-staining in CMV-EBV-, CMV+EBV-, CMV-EBV+ and CMV+EBV+ (left to right) children.

Values indicated percentage of total CD8⁺ T-cells expressing early (CD27⁺CD28⁺), intermediate (CD27⁺CD28⁻), and fully differentiated (CD27⁻CD28⁻) phenotypes. Below are representative flow cytometry plots from individual donors of CD27 (y-axis) and CD28 (x-axis) co-staining of the total CD8⁺ T-cell populations in CMV-EBV-, CMV+EBV-, CMV-EBV+ and CMV+EBV+ (left to right) Gambian children.

4.3.5 Interferon gamma (IFN- γ) responses to EBV lytic and latent-cycle antigens in Gambian children

Next, identifying and measuring the size of the responses to immunodominant epitopes examined the impact of EBV infection in childhood on the CD8⁺ T-cell response to this virus. In order to describe the immunodominant responses in Gambian infants, IFN- γ ELISPOT assays were performed using 21 peptide pools of between 10 and 11 individual peptides combining 63 latent and 43 lytic-cycle epitopes, covering a broad range of HLA types (Methods section 2.12, Tables 2.4 and 2.5). Briefly a two dimensional matrix was constructed using the 21 pools, with each peptide appearing in two pools only. Deconvolution of the matrix enabled identification of the individual peptide that the donor was likely to be responding to. The pools contained peptides from only one protein whenever practically possible. Where this was not the case responses have been expressed as coming from either protein. Although the assays were conducted with PBMCs, the peptides used focused on known CD8⁺ T-cell epitopes including those restricted through relevant Gambian alleles, A*0201, B*0801, B*3501, B*5301 and B*5801, as previously reported ((Allsopp et al., 1992) Louis Marie Yindom, unpublished data). The cell numbers acquired from these children were limited and therefore CD4⁺ T-cell depletion was not practical. Responses were regarded as positive if they were if >3 times the mean of the quadruplicate negative control wells on the plate and ≥ 50 SFU/10⁶ PBMCs. If the negative background wells had ≥ 30 SFU/10⁶ or the positive control wells were negative the assay would have been classified as 'failed', although this was not the case for any of the donors screened (Methods Section 2.12).

Work on adult Caucasian donors has identified a range of CD8⁺ T-cell restricted epitopes. The lytic antigen responses tend to be focused on IE and E proteins of which there are 13, 8, 4

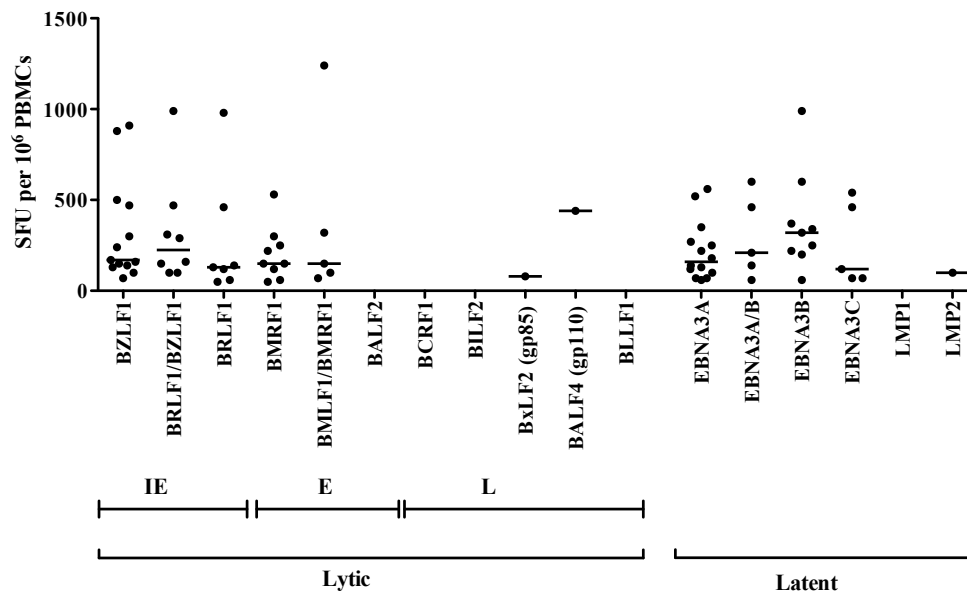
and 6 peptides included to BRLF1, BZLF1, BMLF1 and BMRF1 proteins respectively. The L lytic cycle expressed antigens generally induce weak responses but five L proteins with 1-4 epitopes per protein have been included in this panel. For the latent antigens, strong responses are detected to the EBNA3A, 3B and 3C proteins of which between 8 and 14 peptides are included in the panel for each protein. LMP-2 is generally a sub-dominant antigen, but contains many epitopes, which were included in the panel. LMP-1 is a poorly recognised target of which six epitopes were included. This pattern of immunodominance has been demonstrated in adult Caucasian donors. It is not known what children in the setting of The Gambia will predominantly respond to.

Out of the 114 children enrolled in the study 96 (84%) children at the visit one time point had sufficient cell numbers to perform IFN- γ ELISPOT assays. Of these, 58 (60%) were IgM-IgG⁺ and 38 (40%) IgM-IgG⁻ donors, these were included as controls. As expected all IgM-IgG⁻ donors produced no IFN- γ in response to EBV peptide pools in the ELISPOT assay. There were 38 (66%) out of the 58 IgM-IgG⁺ children screened that produced an IFN- γ response to at least two EBV peptide pools in the matrix (corresponding to one peptide), and 20 (34%) that were non-responders (did not produce a response to any pool lytic or latent). Of these responders, while nine (24%) and five (13%) responded to either the lytic or latent pools respectively, the majority, 24 (63%), responded to both. Figures 4.7 and 4.8 illustrate the pattern of immunodominance identified in the 38 IgM-IgG⁺ Gambian children that responded. The late protein BALF4 had a single responder with a high magnitude response (440 SFU/10⁶). For the latent antigens, the highest responses were seen to EBNA 3A, 3B and 3C proteins, with none detected to LMP1. EBNA3B responses were of the highest magnitude among responders to latent proteins (Median 320 SFU/10⁶). Interestingly, there was no

significant difference in the size of response to lytic versus latent peptides ($p=0.07$). However, there were more IgM–IgG+ children that did not respond to the latent pool but responded to the lytic pools ($n=15$) than children that did not respond to the lytic pool but responded to the latent pools ($n=5$) (Figure 4.7 B). The magnitude of the response did not correlate with the percentage of children that responded (Figure 4.8). For example EBNA3A was the most recognised latent antigen (22% of children) however it did not elicit the highest response (Median 160 SFU/ 10^6) (Figure 4.7A and 4.8).

Assessment using intracellular cytokine staining and flow cytometry analysis was performed to determine whether there was prominent production of cytokines other than IFN- γ or markers of degranulation, after overlapping peptide stimulation. There were very few donors identified as responders to overlapping peptide pools of lytic protein, BZLF1, and latent protein, EBNA3A, therefore the results of this are detailed in Appendix I. With the caveat of a small sample size, the magnitude and proportion of children that had any detectable PBMC response to antigen stimulation did not significantly differ between lytic and latent protein stimulations.

A.



B.

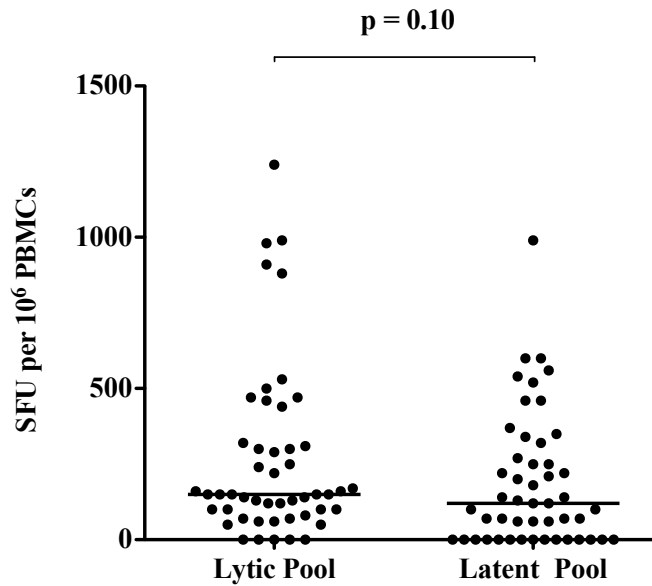


Figure 4-7 IFN- γ ELISPOT responses from PBMCs stimulated with lytic and latent-cycle antigens in Gambian children.

Size of IFN- γ (SFU per 10^6 PBMCs) response produced following antigen stimulation with pools of known CD8⁺ restricted peptides expressed according to A. proteins and B. lytic and latent cycle epitopes.

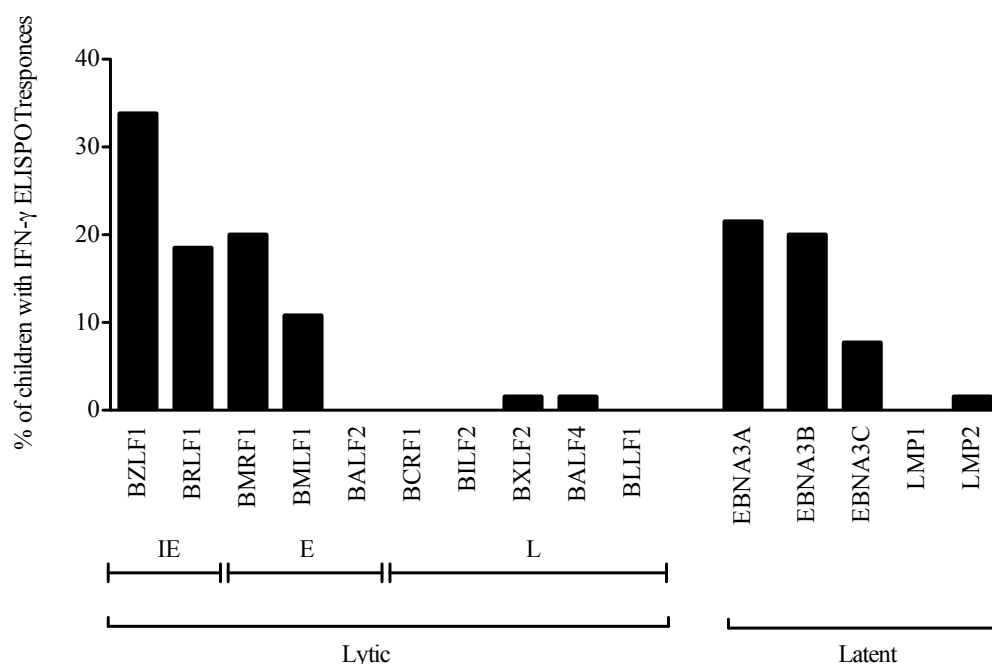
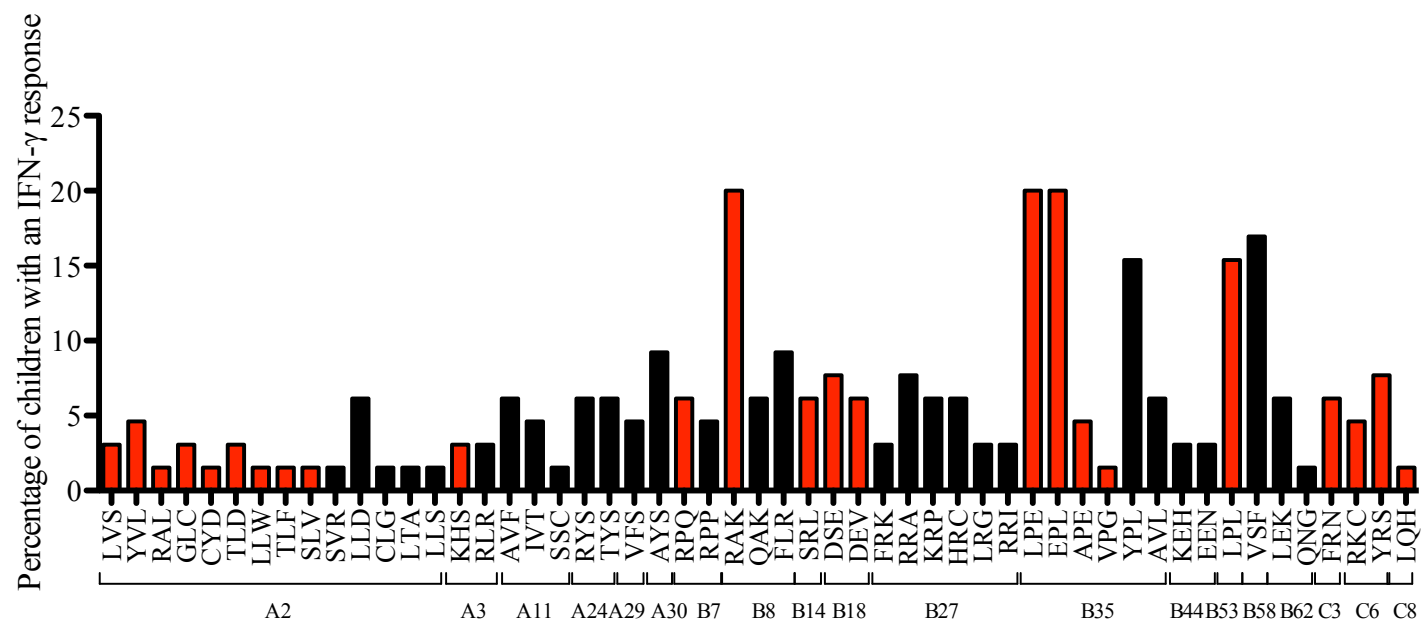


Figure 4-8 *Percentage of children with PBMCs producing IFN- γ in response to antigen stimulation with pools of CD8+ restricted peptides.*

There were 20 IgM–IgG+ children that produced no response to the peptide pools and this could be because they do not have an HLA type of interest. Although the HLA types of these children were not known, previous data from The Gambia give HLA frequencies of 25%, 17%, 24%, 18% and 15% for HLA A*0201, B*0801, B*3501, B*5301 and B*5801 alleles respectively (Louis-Marie Yindom, unpublished data) (Allsopp et al., 1992). Figure 4.9 shows the percentage of children with IFN- γ ELISPOT responses for each of the likely individual lytic and latent peptides that were responded to in the peptide pools following deconvolution of the two-dimensional peptide matrix, alongside their HLA restrictions. Most of the responses are to epitopes restricted through alleles known to be of high frequency in The Gambian population. However, there were not many A*0201 responders despite this being an allele of relatively high frequency in the population.



Lytic (red) and Latent (black) epitopes groups by HLA restriction

Figure 4-9 *Percentage of children producing an IFN- γ ELISPOT response from PBMCs stimulated with known CD8+ restricted lytic (red) and latent cycle (black) pooled epitopes.*

4.3.6 EBV-specific responses in IgM–IgG+ children visualised with tetra

To further analyse the impact of EBV infection in this cohort MHC class I was used to identify and analyse the surface marker phenotype of epitope specific CD8+ T-cells. From the aforementioned IFN- γ ELISPOT data we selected the following epitopes: B*0801 RAKFKQLL, B*3501 EPLPQGQLTAY and A*0201 GLCTLVAML for peptide manufacture, as they were frequent targets of the immune response. Tetramers were then tested for specificity against HLA-matched and mismatched seropositive and seronegative donors (Figure 4.10). There was some non-specific background staining noted in the mismatched and seronegative donor for B*0801 RAKFKQLL, but a clearly defined CD8+ tetramer positive population was present in the HLA matched donor only.

Fifteen IgM–IgG+ children identified as responders to the above three epitopes in the previous section were further tested by tetramer staining to determine the phenotype of epitope specific CD8+ T-cells compared to the total CD8+ T-cell population. Alongside tetramer staining where cell numbers permitted (n=12), individual IFN- γ ELISPOT assays were performed using the same peptide epitopes (B*0801 RAKFKQLL, B*3501 EPLPQGQLTAY and A*0201 GLCTLVAML), as a single antigen stimulus providing a functional readout to the tetramer response detected. Figure 4.11 shows that the IFN- γ ELISPOT response was positively correlated with the number of tetramer positive cells, although the frequencies were much higher by tetramer staining suggesting that they may be secreting cytokines other than IFN- γ or be non-functional. Out of the subset of 15 children tested by tetramer staining four were CMV negative. One donor dropped out at the visit time point and therefore could not be included in the analysis of phenotypic changes over time.

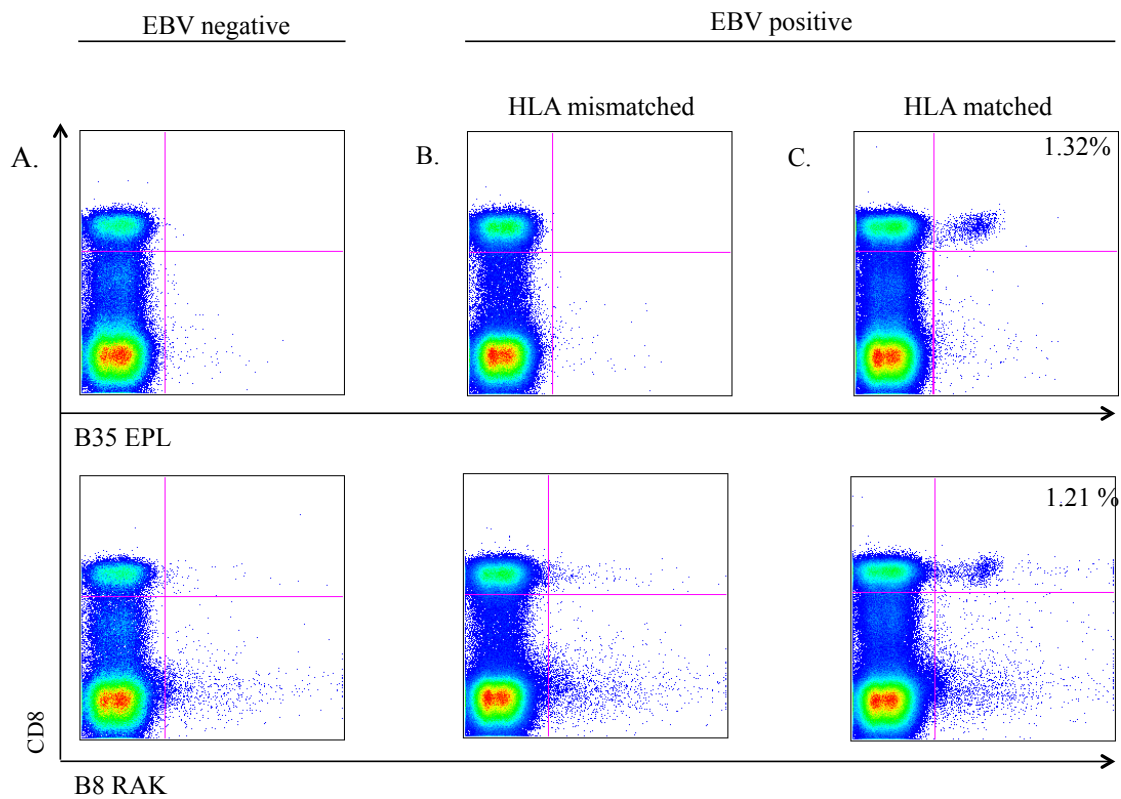


Figure 4-10 Example of tetramer staining for B35 EPLPQGQLTAY (BZLF1) and B8 RAKFKQLL (BZLF1) on seronegative (A), seropositive HLA mismatched (B) and HLA matched (C) donors.

Percentage indicates tetramer as a proportion of CD8+ T-cells.

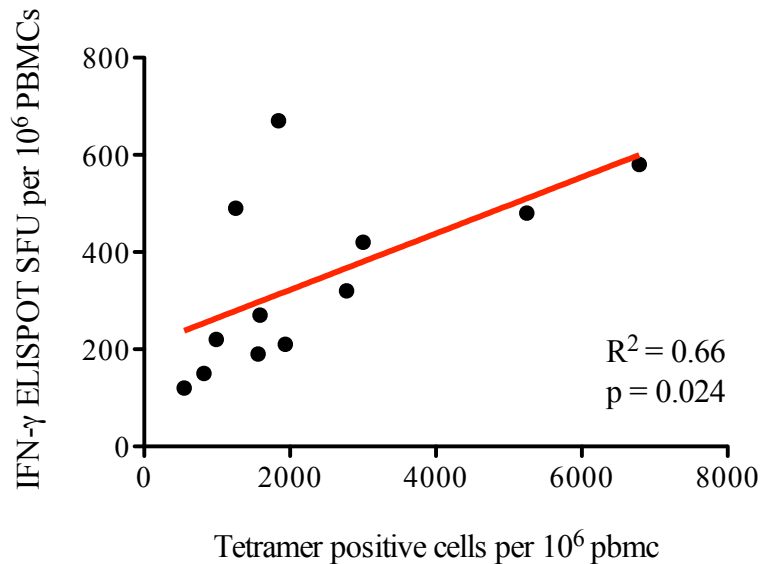
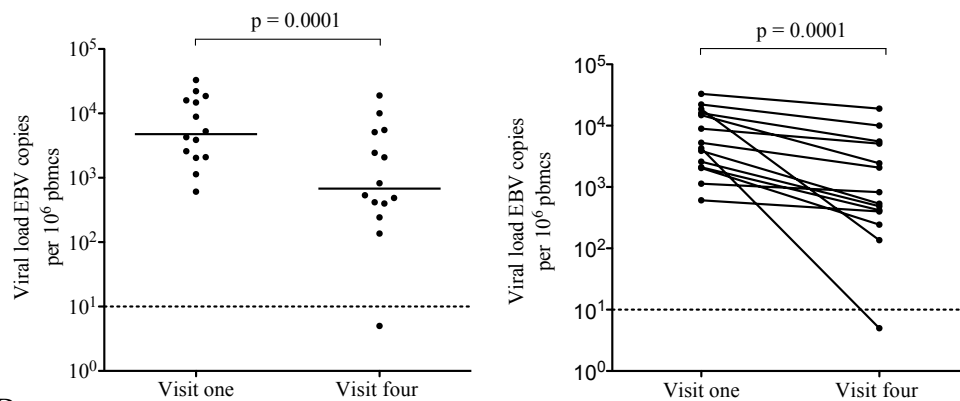


Figure 4-11 Number of tetramer positive cells (x-axis) correlates with IFN- γ production (y-axis) in ELISPOT assays stimulated with the single epitopes corresponding to tetramers used, B*0801 RAKFKQLL, B*3501 EPLPQGQLTAY or A*0201 GLCTLVAML.

As there was an observed decrease in EBV viral genome loads in donors sampled at visit one and visit four time points, the subset of 15 donors available for tetramer analysis were further examined to see if this decrease altered EBV-specific CD8+ T-cell proportions over time. As figure 4.12 illustrates, there was a significant decline in both the EBV viral genome load ($p=0.0001$) and percentage of EBV-specific CD8+ T-cells ($p=0.0052$) over the six month time period. By contrast there was no significant difference in the total CD8+ T-cell population over time (data not shown).

A.



B.

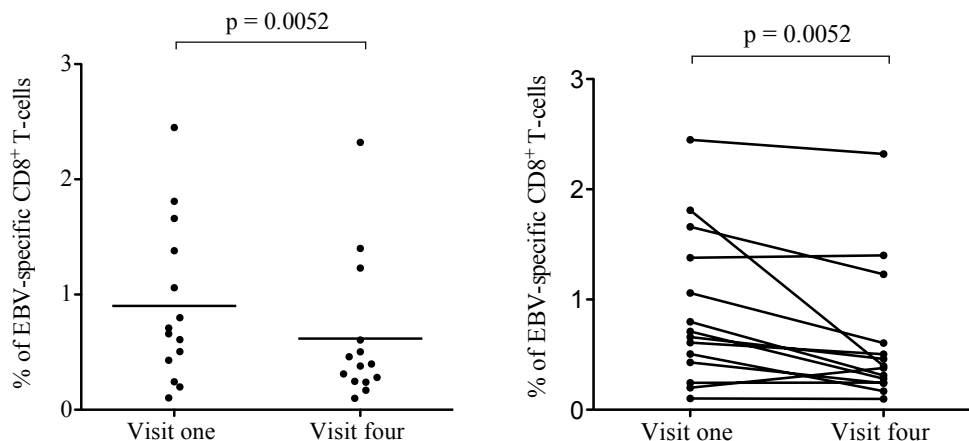


Figure 4-12 EBV genome loads and dynamics over time.

A. EBV genome load (dotted line represents limit of detection of the assay) and B. percentage of EBV-specific CD8+ T-cells at Visit one (Day 0) and Visit four (six months) later in IgM–IgG+ Gambian children. Line and symbol graphs (right column) illustrate the dynamics of each individual's response over time.

As these donors showed virological evidence of recent infection, EBV-specific CD8⁺ T-cells were examined for evidence of recent activation. Phenotyping of the EBV-specific CD8⁺ T-cells detected using Class I tetramer staining was also performed on this subset of IgM–IgG⁺ Gambian children (n=15). The following markers, CD38, HLADR, bcl-2, ki-67, Perforin, Granzyme B, CD57 and T-bet were assessed allowing for some comparisons to be made with cellular phenotypes seen in Caucasian adult long-term carriers. A comparison of expression of the above phenotypic markers on EBV-specific and the total CD8⁺ T-cell populations in these IgM–IgG⁺ children was performed at the visit one time-point. To further assess whether expression developed or disappeared over time the phenotype at visit one was compared with the phenotype six months later at visit four.

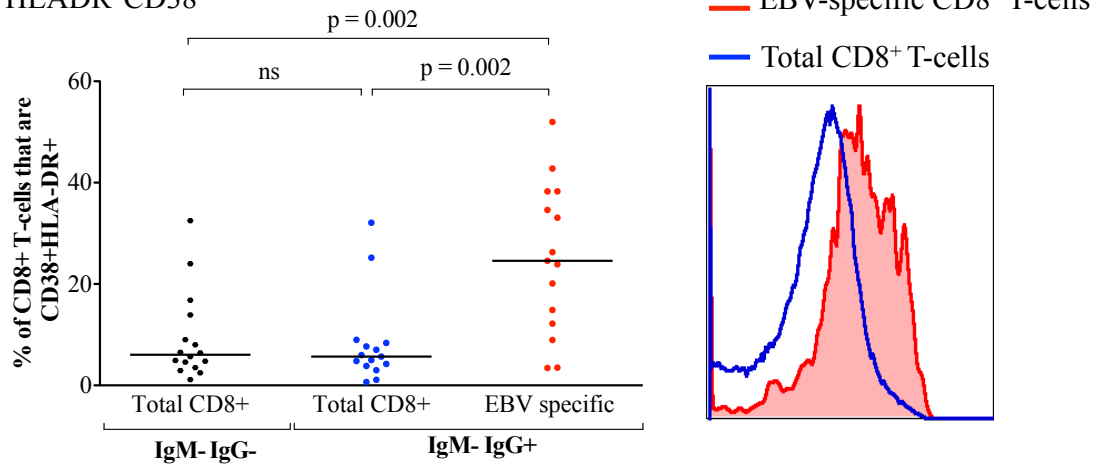
The literature on Caucasian long-term carriers with no history of AIM shows that the EBV-specific CD8⁺ memory T-cells display a resting phenotype, most lack activation markers and express high levels of bcl-2, with low level perforin expression and cytolytic activity. (Callan et al., 1998b, Catalina et al., 2002, Dunne et al., 2002, Faint et al., 2001, Hislop et al., 2001). CD38 was used in combination with HLADR as a marker of activation, as CD38 is expressed on almost all infant T-lymphocytes throughout the first year of life (de Vries et al., 2000). The anti-apoptotic marker bcl-2, marker of cell cycling Ki-67, cytolytic marker Granzyme B and marker of senescence CD57 were also used. In addition, expression of T-bet was also assessed which in relation to HIV infection has been shown to play an important role in CD8⁺ T-cell effector function, with higher levels detected in elite controllers compared to chronically infected progressors (Hersperger et al., 2011). It was therefore of interest to see if this was expressed by EBV-specific CD8⁺ T-cells from children in The Gambia where EBV viral load set-points are known to be higher.

For a subset of these IgM–IgG⁺ children (n=12), where cell numbers permitted a second panel of markers assessing for co-expression of multiple inhibitory receptors (PD-1, 2B4, CD160) on the surface of EBV-specific CD8⁺ T-cells was examined. The co-expression of these markers has recently been correlated with HIV loads, suggesting that they represent an ‘exhausted’ cellular phenotype (Yamamoto et al., 2011, Petrovas et al., 2006, Petrovas et al., 2009). However, higher co-expression of these ‘inhibitory receptors’ also occurs in HIV-2 infected individuals, with largely undetectable viral loads who have polyfunctional Gag responses, and may therefore better reflect cellular differentiation status rather than exhaustion (T de Silva, Unpublished data). This is in keeping with data in healthy adults showing PD-1 expression correlates with differentiation markers and does not inhibit the capacity to secrete cytokines upon stimulation (Duraishwamy et al., 2011). The role these markers might play in EBV infection in childhood is not known and one could hypothesise that they may correlate with the higher EBV viral loads seen in Gambian children if up-regulated on EBV-specific CD8⁺ T-cells.

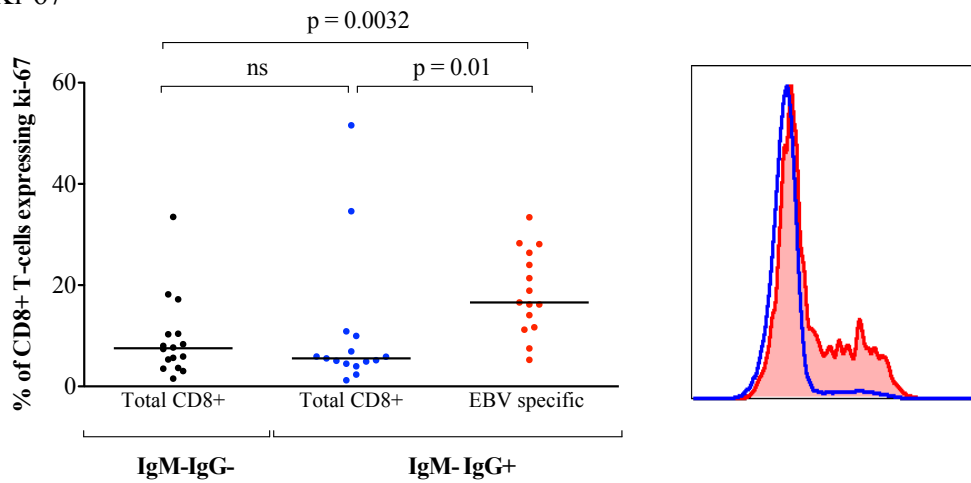
Analysis of activation, proliferation and anti-apoptotic marker expression on EBV-specific CD8⁺ T-cells showed that they were significantly different from the total CD8⁺ T-cell population, with a clear change in expression over time. There were significantly more HLADR⁺CD38⁺ (p = 0.002) and Ki-67⁺ (p = 0.01) EBV-specific CD8⁺ T-cells compared to the total CD8⁺ T-cell population in the same donors, with lower levels of the anti-apoptotic marker, bcl-2, compared to the total CD8⁺ T-cell population in the same donors (p <0.001) (Figure 4.13). A further comparison was made of the total CD8⁺ T-cell populations in the IgM–IgG⁺ group with the total CD8⁺ T-cell populations in a subset of control IgM–IgG[–]

Gambian children (n=16) and no significant differences in the percentage of HLADR+CD38+ and Ki-67+ expressing cells were found. There was however significantly more bcl-2 (p=0.0001) expressed by the total CD8+ T-cell population in the IgM-IgG- children (Figure 4.14). The percentage of HLADR+CD38+ EBV-specific CD8+ T-cells in the IgM-IgG+ children declined significantly over time (p=0.003), whereas cellular expression of bcl-2 significantly increased (p=0.013), with a non-significant decline seen in frequency of cells expressing Ki-67 (Figure 4.14). Of note one donor dropped out at the visit four and therefore it was not possible for them to be included in this analysis.

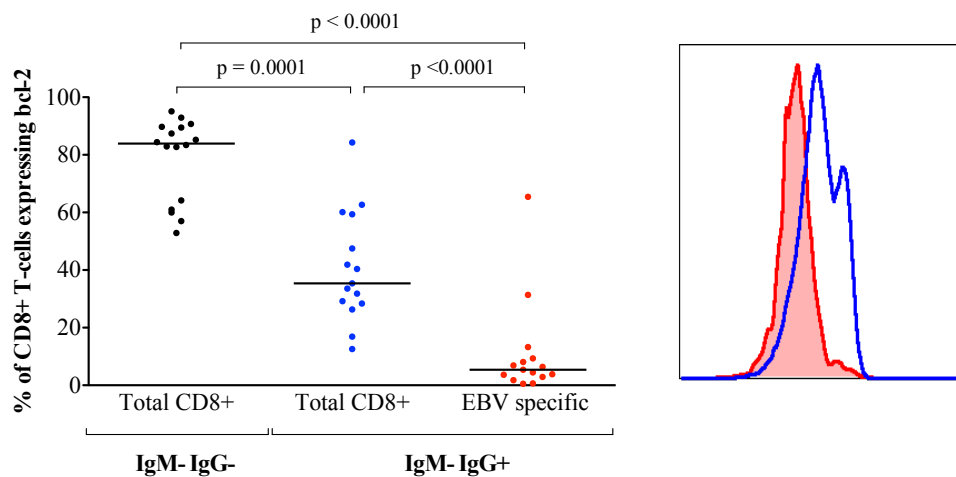
A. HLADR⁺CD38⁺



B. Ki-67



C. Bcl-2

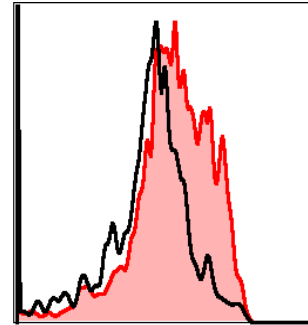
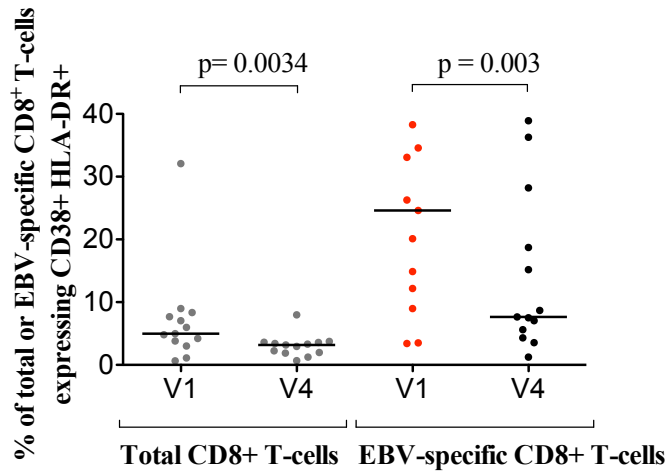


P value calculated using Wilcoxon matched-pairs signed rank test for paired IgM-IgG⁺ donor values, *P* values calculated using the Mann-Whitney U comparison of unpaired data for comparisons between IgM-IgG⁺ and IgM-IgG⁻ donors.

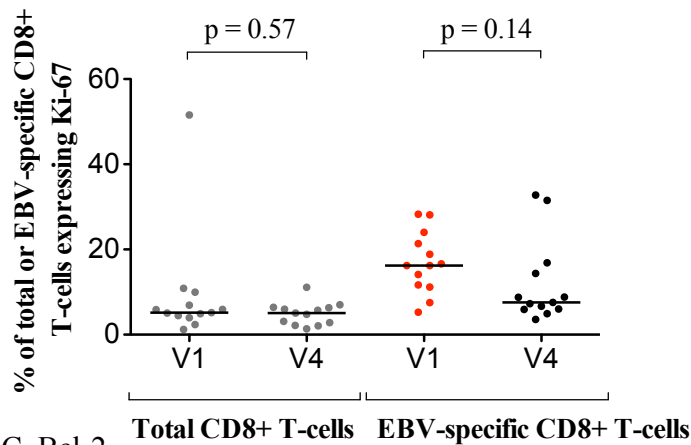
Figure 4-13 *Frequency of cells expressing HLADR+CD38+(A), Ki-67 (B) and bcl-2 (C) on total and EBV-specific CD8+ T-cells from IgM–IgG+ Gambian children (n=14) and total CD8+ T-cells from IgM–IgG– Gambian children (n=16).*

The left columns show percentage of marker positive cells for all donors for the total CD8+ T-cells from IgM–IgG– children (black symbols) and total CD8+ T-cells (blue symbols) and EBV-specific CD8+ T-cells (red symbols) from IgM–IgG+ children. The right hand panels show histograms from a representative donor illustrating the expression of the three markers by EBV-specific CD8+ T-cell (red) and the total CD8+ T-cell population (blue).

A. HLADR⁺CD38⁺

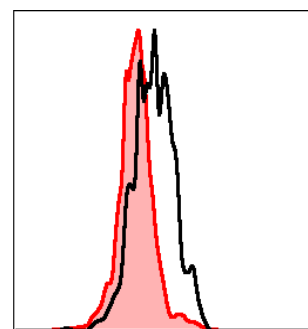
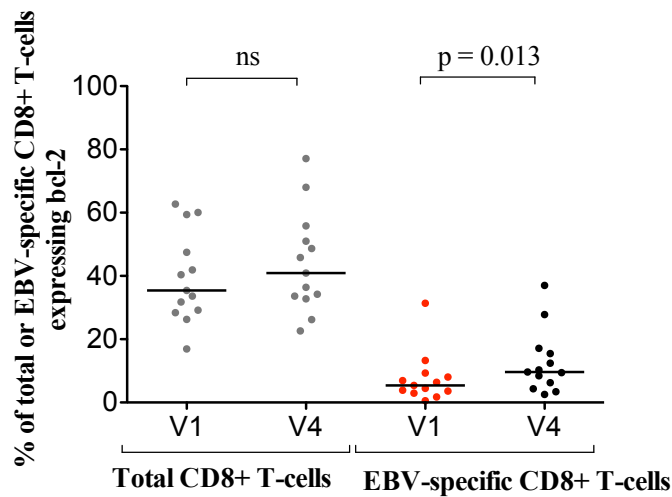


B. Ki-67



— Visit one
— Visit four

C. Bcl-2



P value calculated using the Wilcoxon matched-pairs signed rank test for paired data

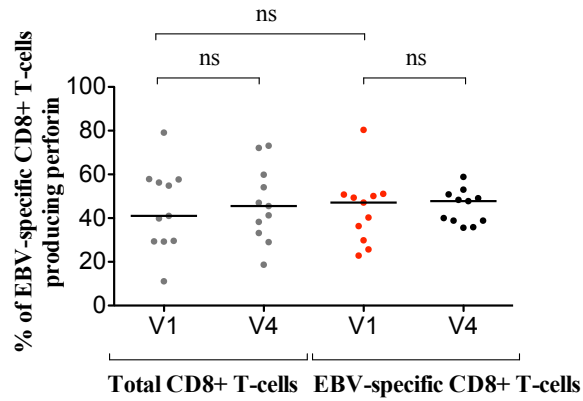
Figure 4-14 Expression of HLADR⁺CD38⁺(A), Ki-67 (B) and bcl-2 (C) by total and EBV-specific CD8⁺ T-cells from IgM-IgG⁺ (n=13) Gambian children over a six-month time period.

The left columns show percentage of marker positive cells for all donors for the total CD8+ T-cells (grey symbols) and EBV-specific CD8+ T-cell (red and black symbols) from IgM–IgG+ children at visit one and visit four, six months later. The right hand panels show histograms from a representative donor illustrating the expression of the three markers by EBV-specific CD8+ T-cell at visit one (red) and visit four (black).

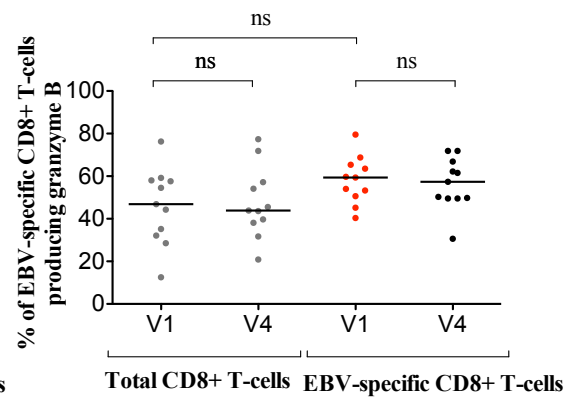
Analysing markers associated with effector function namely perforin, Granzyme B, T-bet and CD57 showed there was no difference in expression among the EBV-specific CD8+ population compared to expression on the total CD8+ T-cell population. For the majority of these phenotypic markers, perforin, Granzyme B and T-bet, there was also no change over time by the total or EBV-specific CD8+ T-cells. However, CD57 expression was significantly up regulated on the EBV-specific CD8+ T-cells over time ($p = 0.04$) but not on the total CD8+ T-cell population (Figure 4.15).

Analysis of markers associated with inhibition of T-cell function on EBV-specific CD8+ T-cells from Gambian children showed they were up regulated in comparison to the total CD8+ T-cell population and remained elevated over time (Figure 4.16). There was no correlation between the percentage of EBV-specific CD8+ T-cells expressing all three of these multiple inhibitory co-receptors and IFN- γ production from PBMCs in response to single peptide stimulation, viral genome loads and CD8+ T-cell differentiation status (data not shown).

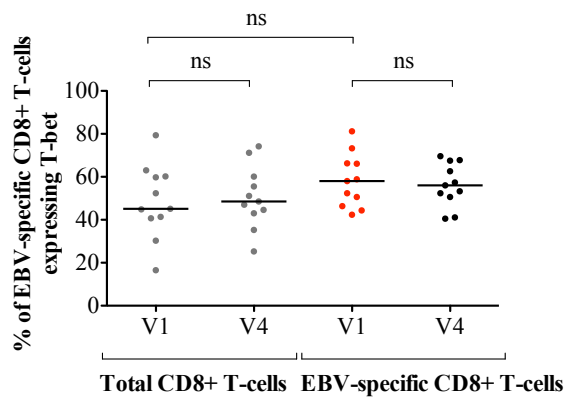
A. Perforin



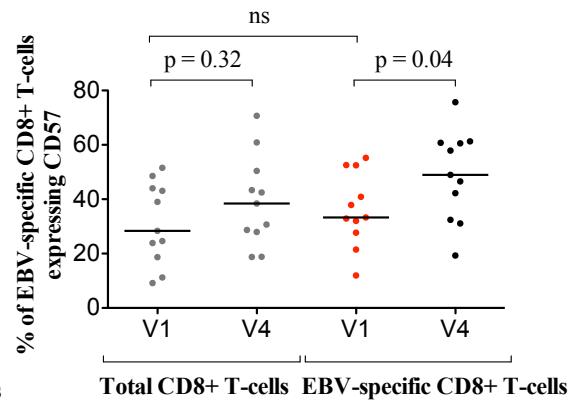
B. Granzyme B



C. T-bet



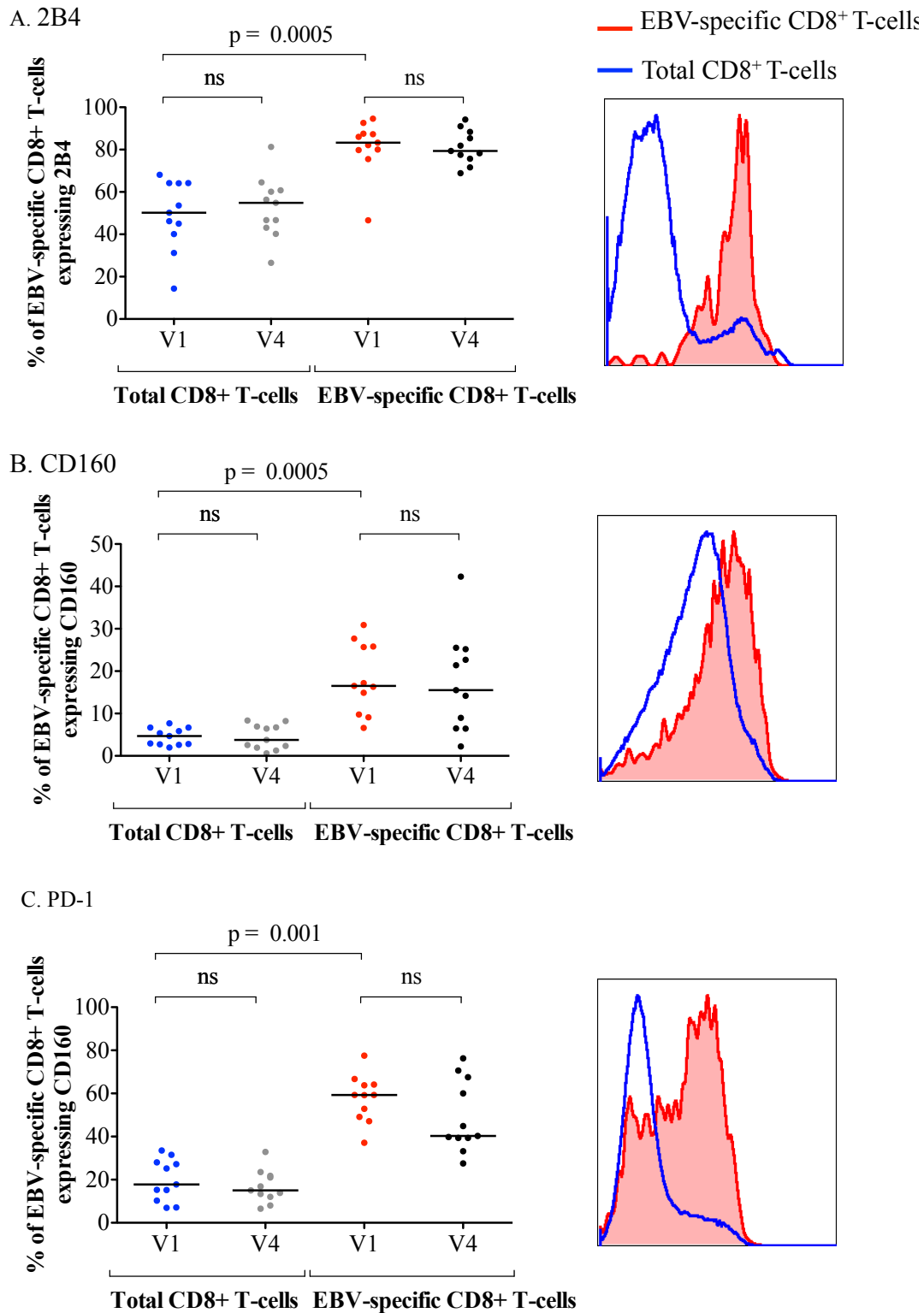
D. CD57



P value calculated using the Wilcoxon matched-pairs signed rank test for paired data

Figure 4-15 *Expression of T-bet and CD57 and production of perforin and Granzyme B on total and EBV-specific CD8+ T-cells from IgM–IgG+ children (n=11) over a six month time period.*

Graphs of cytolytic markers Perforin (A) and Granzyme B (B), T-box transcription factor, T-bet (c) and marker of senescence CD57 (D) in total CD8+ T-cells (grey) and EBV-specific CD8+ T-cells at visit one (red) and visit four (black).



P value calculated using the Wilcoxon matched-pairs signed rank test for paired data

Figure 4-16 Expression of CD160, PD-1 and 2B4 on total and EBV-specific CD8+ T-cells from IgM-IgG+ children ($n=11$) over a six month time period.

Graphs (left column) of 2B4 (A) CD160 (B) and programmed death-1, PD-1, (C) expressing cells in the total CD8+ T-cell (grey) and EBV-specific CD8+ T-cell populations at visit one (red) and visit four (black). Histograms on the right from a representative donor illustrating expression of 2B4 (A), CD160 (B) and PD-1 (C) on total CD8+ T-cells (blue) and EBV-specific CD8+ T-cells (red).

This completes the section relating to IgM–IgG+ children who are likely to have been infected with EBV in the last six months to a year. The following data set refers to children with serological evidence, IgM+IgG –/+ of recent infection.

4.3.7 EBV VCA IgM+IgG-/+ Children: Size of lymphocyte populations and EBV loads in PBMCs

Six children in total were identified as being EBV VCA IgM+IgG-/+ out of 114 children screened: five identified in Table 4.2, and a further donor ID087 was found to be IgM+IgG-/+ at visit three. Three of these children were identified as IgM+IgG-/+ at visit one: donor ID061 for whom data are available from all three-time points, while donors ID013 and ID053 were both non-responders in IFN- γ ELISPOT assays and therefore no tetramer staining was performed. A further two donors ID007 and ID082 were identified as IgM+IgG-/+ at visit four, but had been seronegative at visit one and finally donor ID087 who was identified at visit three was seronegative at visit one (there were no cells available at the visit three time point for this donor). The serological IgM+ findings suggest that these children were more recently infected (in the last 120 days) than the IgM-IgG+ children, discussed previously. All were asymptomatic for classical symptoms of acute EBV infection (fever, lymphadenopathy, malaise) at the time of recruitment based on maternal history and clinical evaluation. As these donors showed serological evidence of recent infection, the size of the peripheral B- and T- lymphocyte compartments were examined. Figure 4.17 and 4.18 show lymphocyte subset numbers and viral loads in PBMCs respectively, taken from the time point at which these children were first identified as being IgM+IgG-/. Surprisingly despite serological evidence of recent infection there was no significant distortion in total T- or B-cell populations in these children (Figure 4.17). Furthermore their viral genome loads did not significantly differ from the IgM-IgG+ Gambian children. On comparison with UK Caucasian donors the viral genome loads were significantly higher than past infected UK medical students and equivalent to those seen during AIM (Figure 4.18).

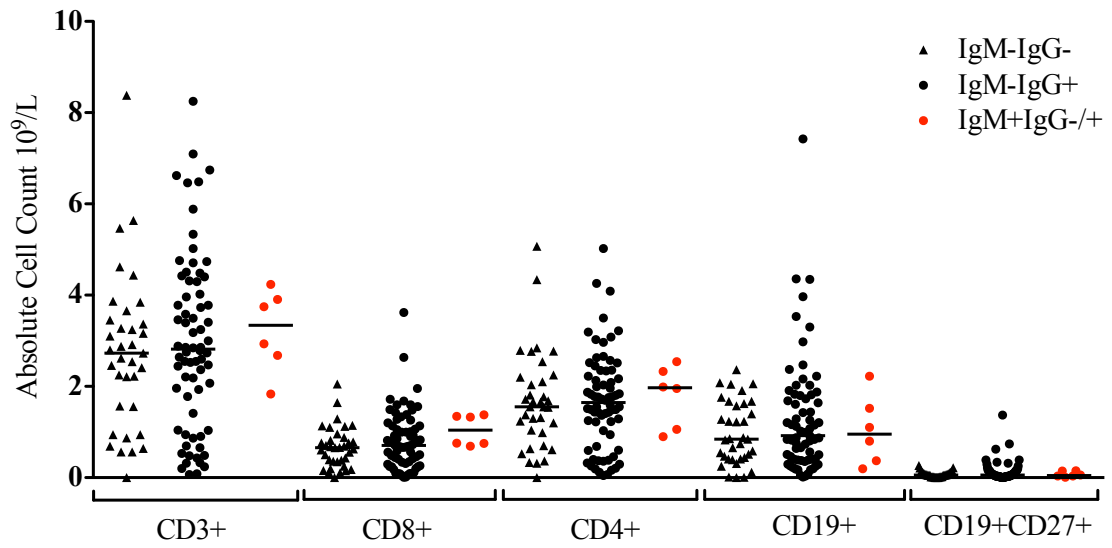
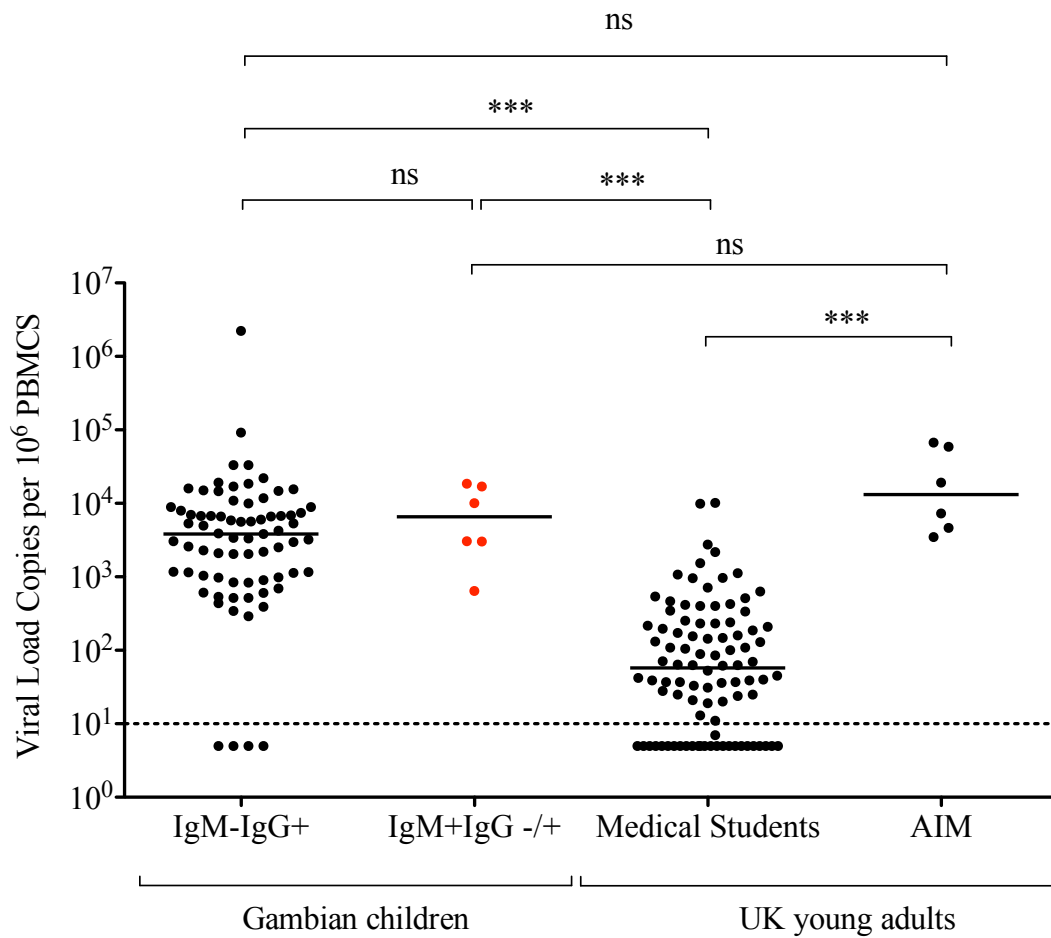


Figure 4-17 Size of lymphocyte populations in the blood of IgM+IgG-/+ ($n = 6$) compared to IgM-IgG+ Gambian children ($n=35$) and IgM-IgG- children ($n=70$). Horizontal lines indicate median number of cells.



P values calculated using Dunn's Multiple Comparison Test (one way analysis of variance (ANOVA)). *** *P*<0.001

Figure 4-18 *EBV genome loads at visit one IgM-IgG+ (n=70) and in IgM+IgG-/+ Gambian children (n=6), adolescent asymptomatic Caucasian medical students (n=94), and Caucasian adults with a diagnosis acute infectious mononucleosis (AIM) (n=6).*

The dashed line represents the lower limit of detection for EBV genomes in the assay. Donors below the dashed line had undetectable viral loads. Horizontal bars indicate the median.

4.3.8 EBV-specific CD8⁺ T-cell responses in IgM+IgG^{-/+} children visualised with tetramers

To analyse the impact of recent EBV infection in this subset of children and explore whether this differed from the IgM-IgG⁺ children, MHC class I tetramers were used to identify epitope specific CD8⁺ T-cells and analyse the surface marker phenotype of these cells. Of the six IgM+IgG^{-/+} donors, ID061, who was HLA*B0801, seroconverted at the first time point and had a detectable tetramer response to the lytic RAK epitope that was monitored serially over time. The other two donors whose seroconversion was detected at the final time point, donors ID007 and ID082, were HLA B*0801 and A*0201 respectively. PBMCs from these donors showed significant EBV-specific CD8⁺ T-cell expansions at visit four, which were noticeably absent at visit one. The last donor ID087 was an HLA B*3501 donor that converted at visit three and again shows evidence of an EBV-specific CD8⁺ T-cell response at visit four with no detectable response at visit one. Figure 4.19 illustrates the EBV-specific tetramer staining of CD8⁺ T-cells for the four aforementioned children's PBMCs. Figure 4.17 shows no significant increase in the total CD8⁺ T-cell count in these IgM+IgG^{-/+} children, however, for donors ID082 and ID007 both had CD8⁺ T-cell percentages that increased from 18.2% to 50.3% and from 19.8% to 40% respectively from visit one to visit four. Thus, significant EBV-specific CD8⁺ T-cell expansions were observed following seroconversion. Donor ID061 had a CD8⁺ T-cell percentage of 41% and a white cell count of $10.8 \times 10^9/\text{L}$ with a lymphocyte count and percentage of $7.29 \times 10^9/\text{L}$ and 67.5% respectively, at the visit one time point.

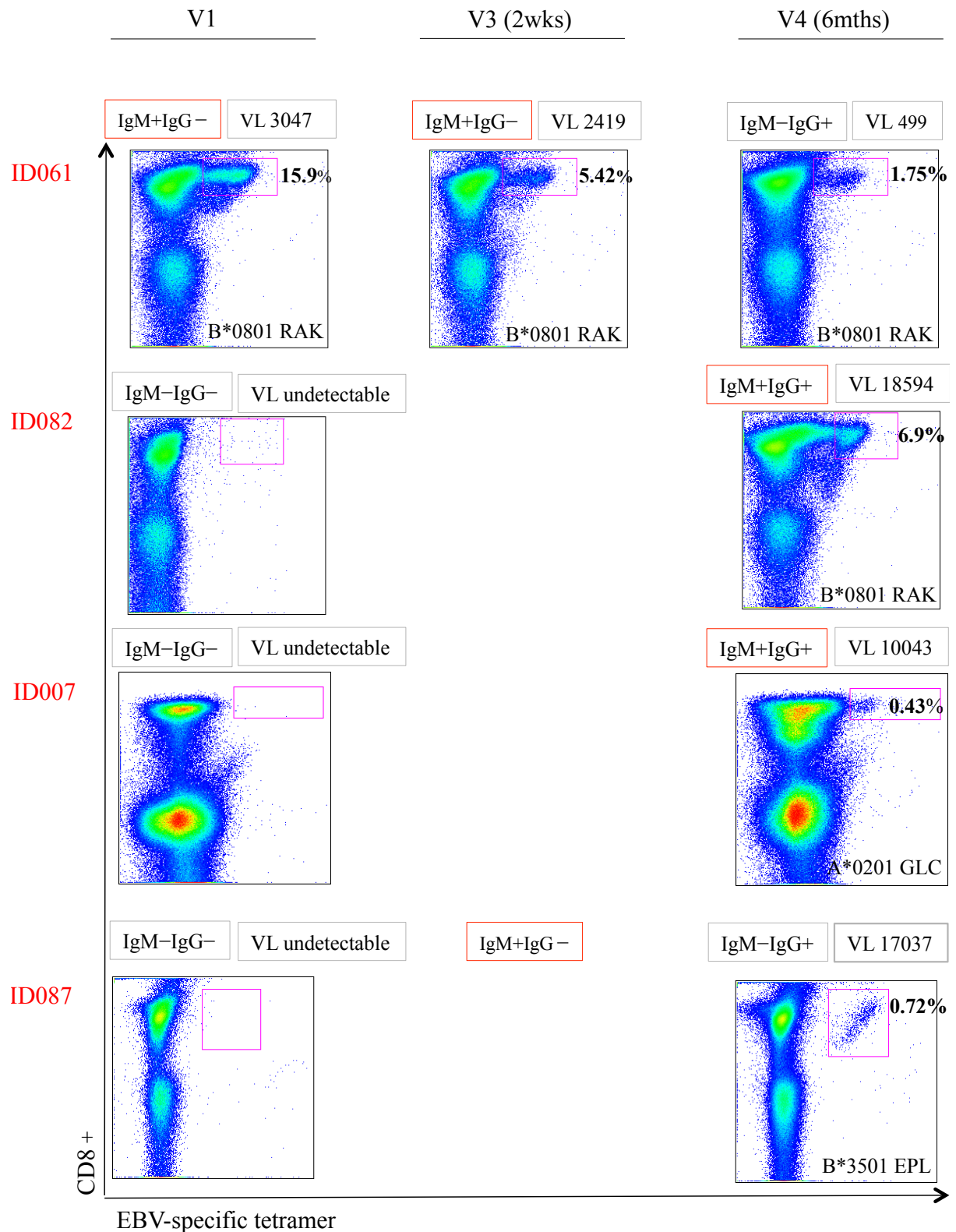


Figure 4-19 Serial EBV-specific CD8+ T-cell staining in IgM+IgG-/+ children.
Time-points are illustrated with sequential viral loads (grey boxes) and serological status (grey boxes), with time point of detection of IgM positivity highlighted (red box).

Where cell numbers permitted, phenotyping of the EBV-specific CD8⁺ T-cells using the previously described panel was performed on stored PBMCs from IgM+IgG^{-/+} children. Unlike the IgM-IgG⁺ children, the total CD8⁺ T-cell population demonstrated high levels of generalised activation as illustrated by Figure 4.20, with 18%, 22% and 49% of total CD8⁺ T-cells expressing activation markers in donors ID061, ID082 and ID007 respectively. Focusing on the EBV-specific CD8⁺ T-cells in these IgM+IgG^{-/+} children at the time of seroconversion, these expressed markers associated with activation, where up to 85% of tetramer+CD8⁺ T-cells co-expressed HLADR and CD38 at their IgM⁺ time-points (Figure 4.21). Donor EBV-specific CD8⁺ T-cells at the time of seroconversion also expressed high levels of Ki-67 (Figure 4.22). Ki-67 expression was higher in acute compared to convalescent samples for donor ID061 who had 12-13% Ki-67⁺ when IgM⁺ but only 3% when IgM⁻. Tetramer positive cells also express significantly lower levels of anti-apoptotic factor, bcl-2, compared to IgM-IgG⁺ Gambian children at visit one (p=0.03) consistent with the phenotype described during AIM (Figure 4.23 versus Figure 4.14).

For donor ID061 for whom we had serial samples from IgM+IgG^{-/+} to IgM-IgG⁺ status staining was performed with further markers. The EBV-specific CD8⁺ T-cells were predominantly Granzyme B producing (60%) and expressed T-bet (50%) with minimal perforin production (20%). This did not alter over time. The percentage of cells expressing CD57 slightly increased over time, with 36%, 36% and 44% of EBV-specific CD8⁺ T-cells being CD57⁺ at visits one, three and four respectively. The expression of ‘inhibitory markers’ on the EBV-specific CD8⁺ T-cells mirrored the pattern seen for the IgM-IgG⁺ children, with the tetramer positive cells being of a predominantly CD160+PD-1+2B4⁺ phenotype at visit one and remaining so at visit four, six month later (data not shown).

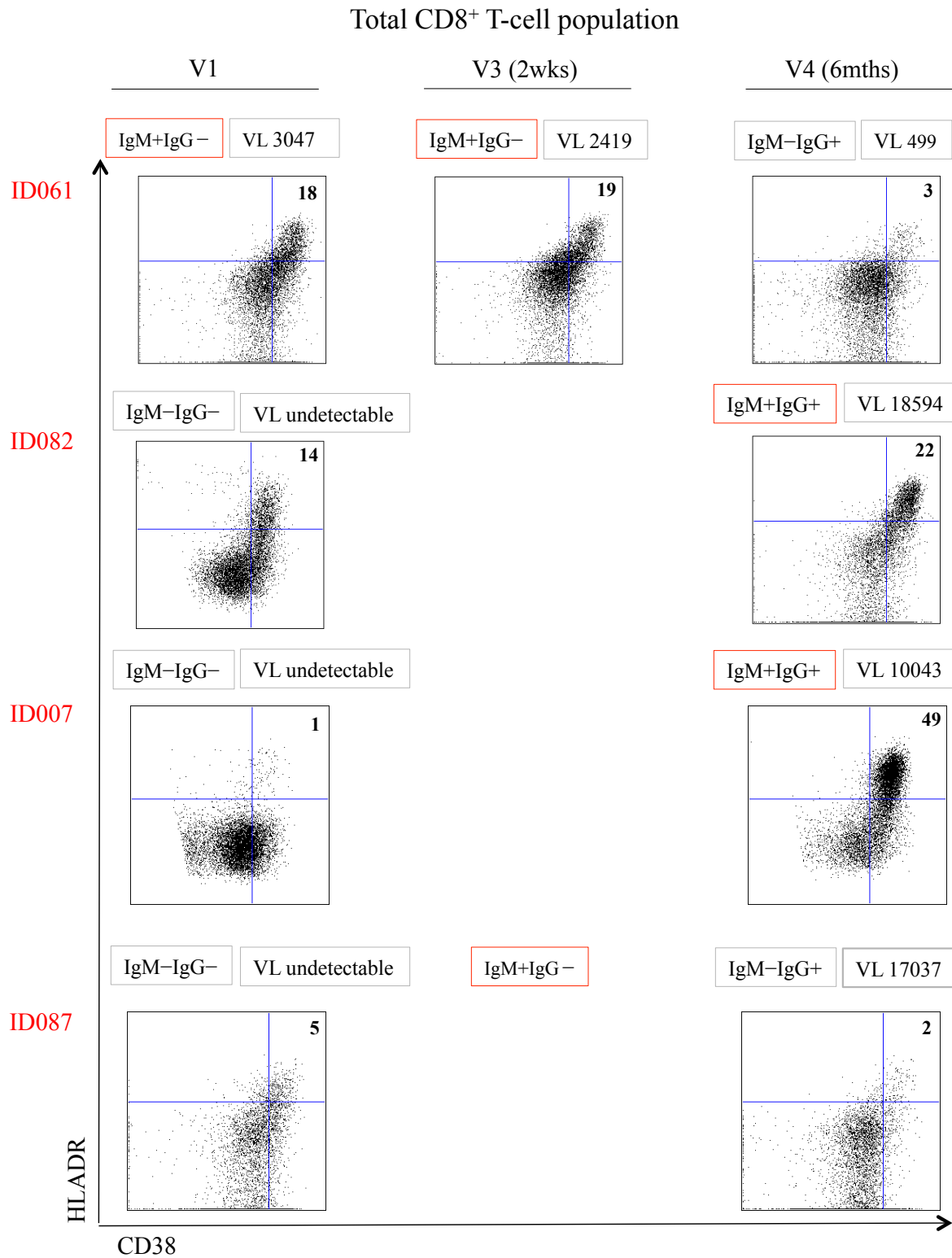


Figure 4-20 *Serial dual expression of activation markers, HLADR and CD38, on the total CD8⁺ T-cell population in IgM+IgG-/+ children.*

Time-points are illustrated with sequential viral loads (grey boxes) and serological status (grey boxes), with time point of detection of IgM positivity highlighted (red box).

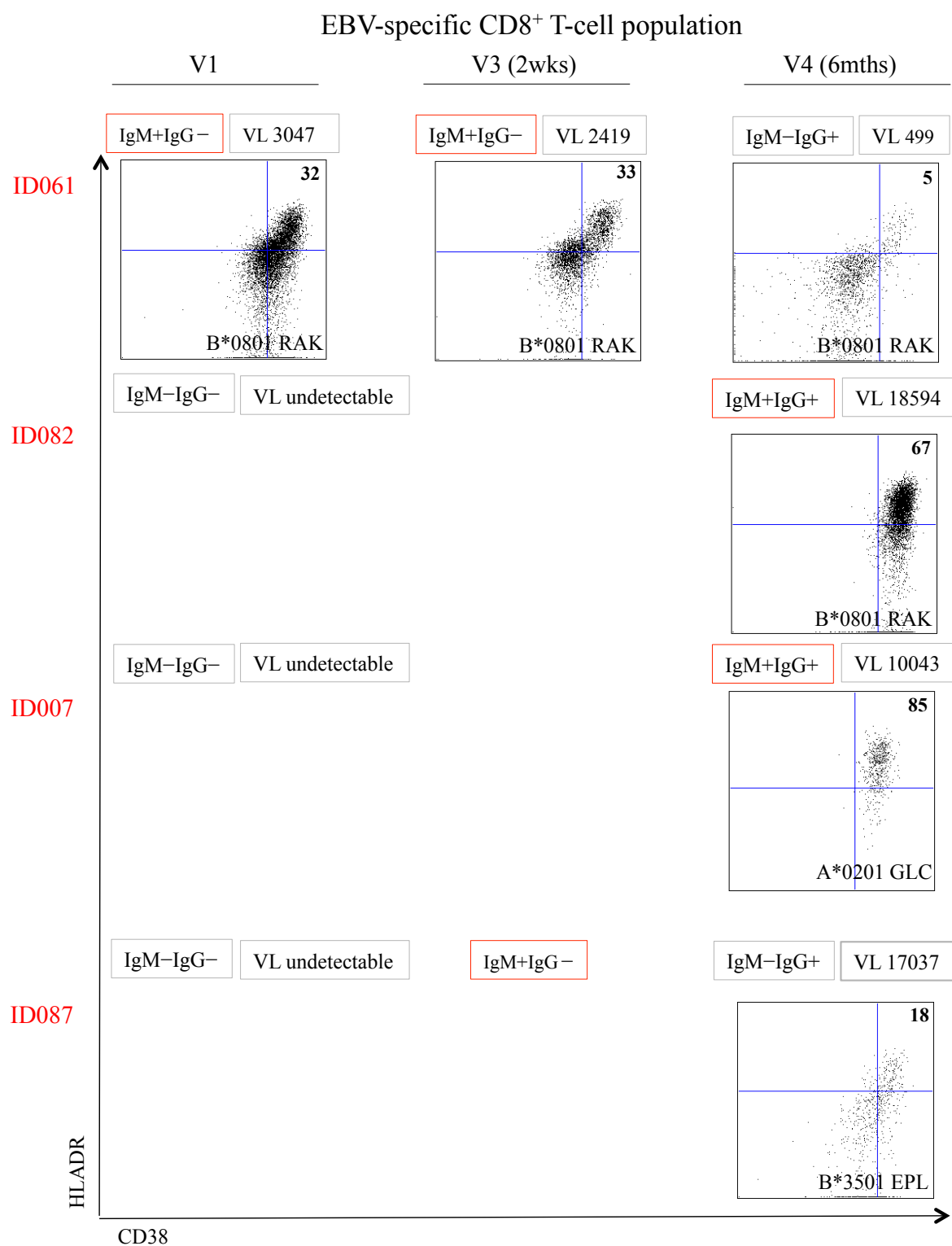


Figure 4-21 *Serial expression of activation markers, HLADR and CD38, on the tetramer EBV-specific CD8⁺ T-cell population in IgM+IgG-/+ children.*
Time-points are illustrated with sequential viral loads (grey boxes) and serological status (grey boxes), with time point of detection of IgM positivity highlighted (red box).

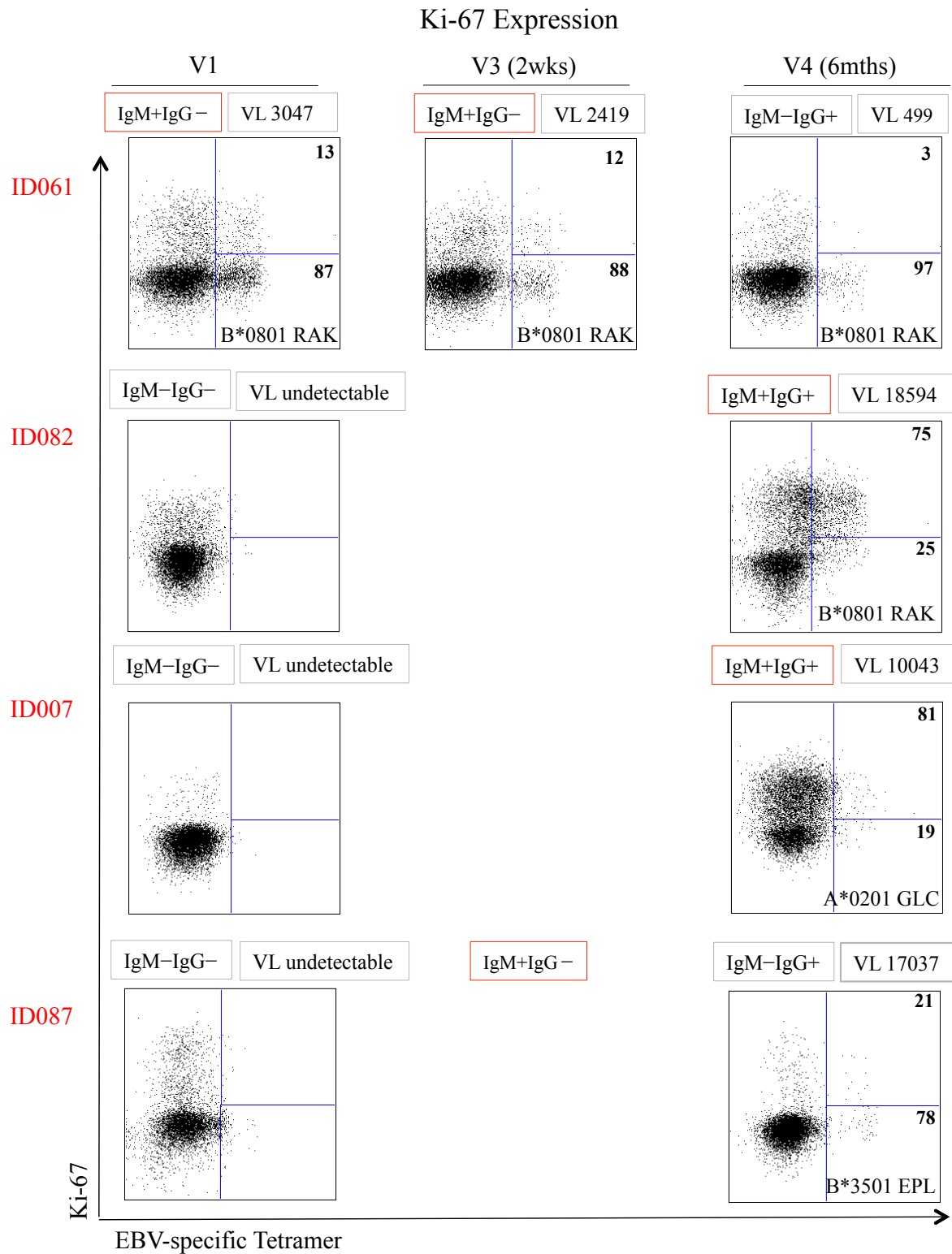


Figure 4-22 Serial expression of cell cycling marker, Ki-67, on the EBV-specific CD8+ T-cell populations in IgM+IgG-/+ children.
 Time-points are illustrated with sequential viral loads (grey boxes) and serological status (grey boxes), with time point of detection of IgM positivity highlighted (red box).

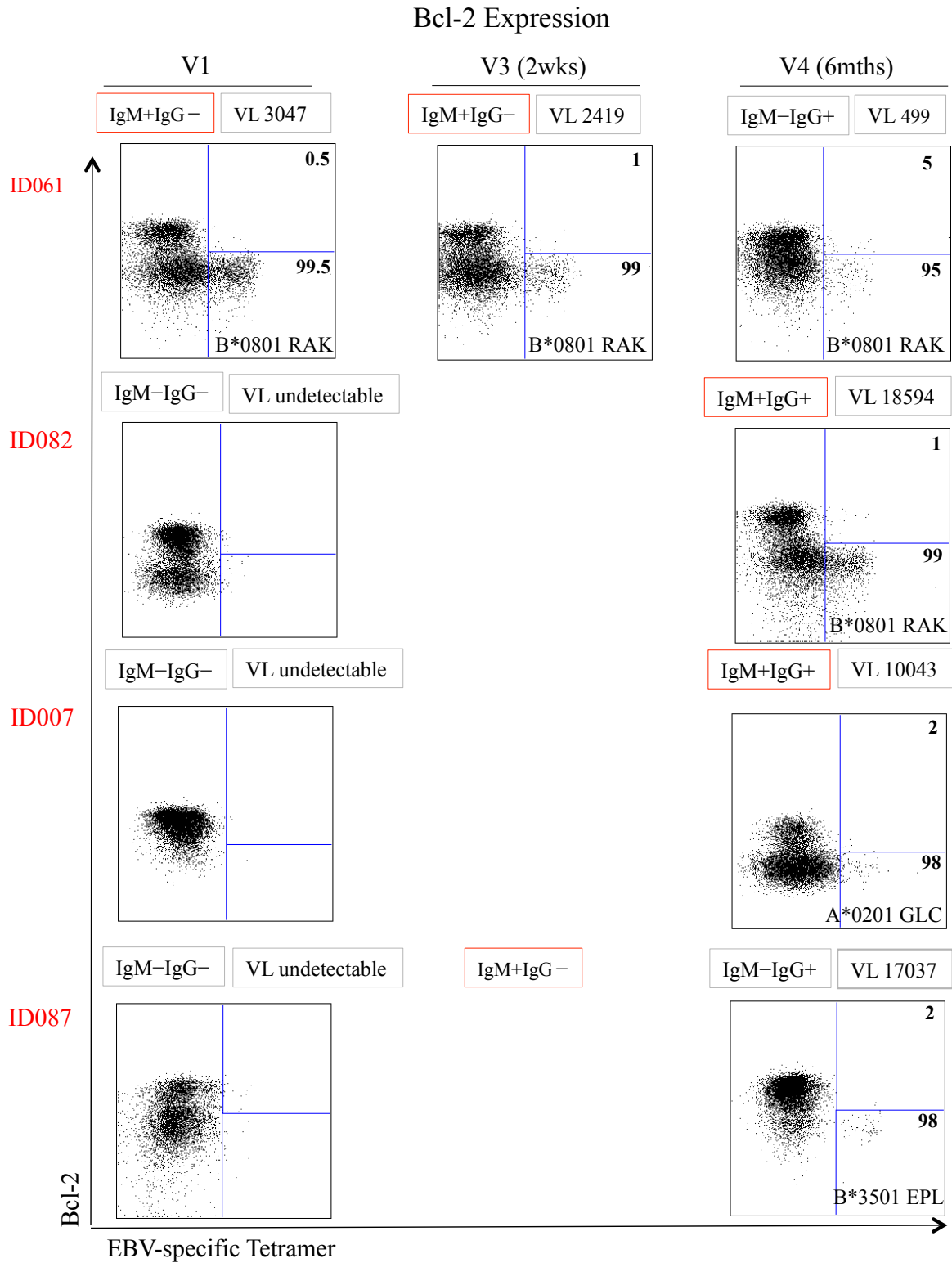


Figure 4-23 Serial expression of anti-apoptotic marker, *bcl-2*, on the EBV-specific CD8+ T-cell populations in IgM+IgG-/+ children.
Time-points are illustrated with sequential viral loads (grey boxes) and serological status (grey boxes), with time point of detection of IgM positivity highlighted (red box).

4.4 Discussion

This study provides a detailed description of EBV-specific CD8⁺ T-cell responses, viral genome loads and IFN- γ responses to known lytic and latent EBV antigens in West African children. Through serial sampling of 114 children in The Gambia aged 14-18 months, 78 (84%) children were identified as being EBV infected or subsequently becoming infected during the six-month study period. There were six children that asymptotically seroconverted during the study period and a subset of a further fourteen recently EBV infected children at Day 0 for whom EBV-specific Class I tetramer staining was performed. This work brings to light some new findings in relation to asymptomatic primary EBV infections in childhood. Firstly, it provides evidence that in subclinical primary infections, there is a lack of the massive peripheral CD8⁺ T-cell response observed in AIM patients, as evidenced by lack of significant lymphocytosis and distortion of total T- and B-cell populations within the blood. Furthermore, EBV-specific CD8⁺ T-cell expansions identified by Class I tetramer staining are of a lower magnitude than has previously been described during AIM (in Caucasian adolescents) despite high EBV viral genome loads and high levels of both tetramer-staining and total activated CD8⁺ T-cells. Secondly, asymptomatic EBV infection in infancy drives differentiation of total and EBV-specific CD8⁺ T-cells with expansion of the ‘intermediately differentiated’ (CD27⁺CD28[–]) CD8⁺ T-cell population rather than the expansion of ‘terminally differentiated’ (CD27[–]CD28[–]) CD8⁺ T-cells observed with CMV infection (Miles et al., 2007). Lastly, the responses to EBV latent antigens were of a similar magnitude to the responses identified to lytic antigens, which contrasts to what has been observed in donors with AIM and healthy UK carriers in whom lytic antigen responses have been shown to dominate (Hislop et al., 2007b).

The six asymptomatic IgM+IgG-/+ children in this study did not have a lymphocytosis, which is consistent with previous literature on silent EBV seroconversion (Biggar et al., 1978a, Fleisher et al., 1979, Silins et al., 2001). Furthermore, they had no distortion in the total CD8+, CD4+, B-cell and memory B-cell counts when compared to EBV IgM-IgG- (uninfected) and IgM-IgG+ (recently infected) Gambian children from the same study site. In the six IgM+IgG-/+ children there was direct evidence of an EBV-specific CD8+ response, which, although broadly speaking, was low compared to AIM donors, was still significant in number and identified with no large total CD8+ expansion. This is in keeping with the hypothesis that EBV is controlled by a primary CD8+ response.

Phenotypically the EBV-specific CD8+ T-cells identified were broadly consistent with what has been described in AIM donors, being highly activated (HLADR+CD38+), in cell cycle (Ki-67+) and pro-apoptotic (low bcl-2 expression) (Callan et al., 2000, Catalina et al., 2001, Dunne et al., 2002, Hislop et al., 2002, Hislop et al., 2005, Tamaru et al., 1993). This is in contrast to what is seen in long-term carriers where EBV-specific CD8+ T-cells display a resting phenotype, lack high levels of activation markers, and express high levels of bcl-2 (Callan et al., 1998b, Catalina et al., 2002, Dunne et al., 2002, Faint et al., 2001, Hislop et al., 2001).

The EBV-specific CD8+ T-cells in IgM-IgG+ EBV-infected Gambian children displayed a highly activated, proliferative and pro-apoptotic phenotype, consistent with recent antigen stimulation. This is likely to be due to these Gambian children all having undergone relatively recent primary infections in the last six-months to a year. This premise is supported by several factors. Firstly, prior epidemiological data from the Sukuta field site has shown that only 18%

of Gambian children are EBV infected at nine months of age (Holder et al., 2010). Secondly, there were high EBV viral genome loads detected in these children, equivalent to those seen in AIM, which declined over the six-month time period and lastly the aforementioned EBV-specific CD8⁺ T-cell phenotypic data from tetramer staining. Furthermore, there is also a reduction in CD8⁺ T-cell activation with a concurrent increase in expression of the anti-apoptotic marker, bcl-2 over time on EBV-specific CD8⁺ T-cells from these recently EBV infected children. The EBV-specific CD8⁺ T-cells in the IgM-IgG⁺ donors had high levels of expression of CD57, a marker usually expressed on highly differentiated cells such as CMV-specific cells, which significantly increased with time. Interestingly, low frequencies of CD57-expressing EBV-specific CD8⁺ T-cells are observed in adult Caucasian populations (Khan et al., 2004, Tan et al., 2000), suggesting that in Gambian children, these EBV-specific cells are more differentiated compared to Caucasians. Although viral genome loads do not necessarily equate to antigen load, given that these children have higher EBV genome loads and thus may have higher levels of antigen stimulation, this may explain why their cells appear more differentiated in terms of CD57 expression. A further analysis of EBV-specific cells using a more extensive panel of differentiation markers (e.g. CD45, CD27, CD28, CD62L, CCR7) would help correlate differentiation status with CD57 expression.

There were two noticeable differences between the IgM+IgG^{-/+} children and the IgM-IgG⁺ Gambian children. The first being the level of CD8⁺ T-cell activation (HLADR+CD38⁺) observed in the total CD8⁺ T-cell population, which was higher in the IgM+IgG^{-/+} (Figure 4.20), but not in the IgM-IgG⁺ infected children, who had similar levels to IgM-IgG⁻ children (medians of 22% (Figure 4.20), 6.5% and 5.6% (Figure 4.13) respectively, $p=0.03$). This could suggest the presence of EBV-specific CD8⁺ T-cells to a predominant epitope(s)

other than that of the Class I tetramers used, or alternatively reflect non-specific by-stander activation of T-cells. During AIM, large expansions of a few monoclonal or oligoclonal CD8⁺ T-cell responses have been shown to dominate (Callan et al., 1996), therefore the activation seen in the total CD8⁺ T-cell population could be EBV-specific. However, expansion and activation of the total CD8⁺ T-cell compartment during heterologous viral infections has also been previously well recognised and herpesvirus specific CD8⁺ T-cells have been shown to consistently contribute to this expansion (Sandalova et al., 2010). Despite the aforementioned acute by-stander activation described, the numbers of heterologous virus-specific T-cells, in the case of CMV and flu, have been shown to remain the same pre- and post- AIM (Odumade et al., 2012).

The second difference was that the IgM–IgG⁺ children did not display an increase in cytotoxic capability as evidenced by the lack of up-regulation of perforin and Granzyme B, but this was observed for one of the IgM+IgG–/+ children, ID061, for which further staining was possible, consistent with the literature on the phenotype of EBV-specific CD8⁺ T-cells seen during AIM (Hislop et al., 2007b).

Primary CMV infection has been shown to significantly alter the differentiation of the total CD8⁺ T-cell compartment (Miles et al., 2007). A significant expansion in the total CD8⁺CD27⁺CD28[–] ‘intermediately differentiated’ phenotype was seen in the recently EBV infected children compared to those that were uninfected. This differs from CMV, which has been shown to drive the majority of CD8⁺ T-cells to a CD27[–]CD28[–] ‘terminally differentiated’ status (Miles et al., 2007). The current data also show that when CMV and EBV co-infection is present the differentiation status of the total CD8⁺ T-cell population

mirrors that seen in CMV infection alone, thus the CMV effect seems to dominate in dual infected children. CMV has been shown to skew the differentiation status of the total CD8+ T-cell population for several years post primary infection (Miles et al., 2008c) and has been implicated in the perpetuation of chronic immune activation (van de Berg et al., 2010), but whether EBV effects are similarly long lasting is not known. Whether the order of acquisition of EBV and CMV infection has any impact on differentiation status also remains to be seen. However, in our setting CMV infection is generally acquired prior to EBV infection, indeed at nine months 66% and 18% of children from Sukuta were infected with CMV and EBV respectively in a previous study (Holder et al., 2010).

In an *in vitro* model, immune activation has been shown to suppress initiation of lytic EBV infection through triggering of the innate immune system specifically via Toll-like receptor-9 (TLR-9), the ligand of which is unmethylated bacterial CpG DNA (Ladell et al., 2007). In African settings levels of immune activation are known to be high secondary to the high prevalence of co-infections such as the previously discussed CMV (Miles et al., 2007), but also malaria, intestinal helminthes and frequent respiratory tract and diarrhoeal infections. African children are therefore likely to have been exposed to a multitude of pathogens in early life, including bacterial CpG, which may *in vivo* influence the nature of early EBV infection in these children by triggering the innate immune system. This could, for example, potentially suppress the initiation of lytic EBV infection via TLR-9 triggering and lead to early establishment of latency. TLR responses in early infancy have been shown to be population dependent and differ between children in North American and Sub-Saharan African populations (Burl et al., 2011, Reikie et al., 2012).

It has been hypothesised that the difference between asymptomatic seroconversion and symptomatic AIM could be related to EBV establishing a different pattern of gene expression during early infection. One possibility is that asymptomatic infection results from an increase in the number of latently infected B-cells in the peripheral blood, whereas during AIM there is more lytic viral replication (Silins et al., 2001). Our data go some way to supporting this notion. Thus IFN- γ ELISPOT responses in our study showed a similar pattern of immunodominance to that seen in Caucasian donors (lytic responses were skewed towards the IE, then E proteins with consistently high latent responses to EBNA3). However, it should be borne in mind that this could be a reflection of the numbers of epitopes screened for each protein, as a greater number of IE and E proteins were screened than late proteins. Interestingly, the lytic and latent IFN- γ responses detected were of a similar magnitude. This is in contrast to what is seen in AIM and in healthy Caucasian long-term carriers where lytic responses are significantly more frequent and of greater magnitude than latent responses (Hislop et al., 2007b). This may be a reflection of the EBV antigens being dominated by viral expression of predominantly latent proteins during acute infection in West African children, although this remains to be confirmed. One way to resolve this would be to look at the virus in peripheral blood during AIM and acute infection in Gambian children using Gardella gel analysis and determine whether the virus is in lytic or latent cycle. It will be difficult to resolve this issue, because in AIM at the time of clinical presentation, the detectable virus in both tonsils and peripheral blood is in latent cycle (Kurth et al., 2000, Tierney et al., 1994). Therefore it is still not known where the EBV lytic antigens are driving the predominantly lytic response that is seen in AIM. It is plausible that by the time patients present with AIM, the lytic burst has been missed or that the virus is harboured in epithelial cells. Studying the

oropharynx, through analysis of oral secretions and assessing viral shedding in the throat may provide a greater insight into early infection in these patients.

There are some limitations to this study, which are worth discussing. Firstly, for the majority of the children in the study, the precise timing of the primary EBV infection cannot be determined. However, from previous data on infants at the same study site, only 18% of infants were found to be EBV infected at nine months of age and from this one can assume that the majority of children would have been infected in the last six-months to a year. Furthermore, the IgM+IgG-/+ could have been infected anytime within the last 120 days and therefore this group could all be at different stages of infection. Secondly, ensuring children are truly asymptomatic in this setting can be challenging as reliance on maternal perception may not be reliable. To combat this, the children all underwent a health screen by the study clinician, including baseline clinical observations such as weight, height, temperature and heart rate and where indicated a rapid malaria test. Recent work by Balfour et al. has suggested that 89% of primary EBV infections in a cohort of University students are in fact symptomatic, although they may not fulfil all classic symptoms of AIM, which is somewhat contradictory to our observations (Balfour et al., 2013). However, older children are more likely to exhibit symptoms. Furthermore, potential confounding, intercurrent infections which could also cause the symptoms screened for (sore throat, lymphadenopathy, fever and fatigue) such as streptococcal pharyngitis, respiratory syncytial virus, parvovirus and adenovirus were not controlled for, and the incidence of symptoms detected in continually EBV negative students was not reported. Lastly, it was not possible to perform HLA typing on all the children studied and therefore HLA mismatching may have skewed the responses that were detected. However to minimise this multiple known epitopes were screened providing a broad

HLA coverage. Furthermore the children studied responded to HLA types which are known to be of a high frequency, A*0201, B*0801, B*3501 and B*5801, in The Gambian population (Allsopp et al., 1992).

These data describe a lack of the typical lytic dominant immune responses seen in AIM with more conservative EBV-specific CD8⁺ T-cell expansions and minimal distortion of the total T- and B-cell compartments despite high levels of circulating EBV genome loads and activated EBV-specific CD8⁺ T-cells. The large atypical total CD8⁺ T-cell expansions seen during AIM did not occur in these IgM+IgG^{-/+} Gambian children despite high detectable EBV viral genome loads. This work further supports the notion that AIM is an immunopathological disease and that symptoms are caused by the significantly expanded CD8⁺ responses to the virus rather than the virus per se (Silins et al., 2001). The lack of a predominantly high lytic antigen response compared to latent reactivity in these children suggests that the form of EBV infection may also differ during asymptomatic infection in childhood compared to AIM. It would be of further interest to explore the tetramer responses to latent antigens in these children, for example using a B*0801 latent epitope such as FLRGRAYGL or QAKWRLQTL MHC Class I tetramer to identify whether latent EBV-specific CD8⁺ T-cell responses are of an equivalent magnitude and phenotype as those detected to lytic epitopes in these children.

Future work looking at the gene expression of the virus during asymptomatic infection in African children would directly identify whether the virus is in a lytic or latent form. Although case identification would be challenging, a comparison of viral gene expression using RT-PCR in adolescent symptomatic and asymptomatic EBV seroconverters in a

developed country setting may provide further insights into whether the form of EBV infection varies depending on age of infection, and how this might relate to the clinical presentation of primary EBV infection. Assessment of oropharyngeal infection analysing virus shedding through throat washings may provide an idea of how the immune response is primed in early infections and whether this differs with age.

CHAPTER 5 : CONSEQUENCES OF EBV INFECTION IN INFANCY: ANTIBODY RESPONSES TO VACCINE ANTIGENS FROM DIPHTHERIA, TETANUS, PERTUSSIS, *HAEMOPHILUS INFLUENZAE B* AND HEPATITIS B

5.1 Introduction

There are very few data in the literature on how chronic viral infections might alter cellular and humoral responses to vaccination or co-infection. The work of Miles *et al.* demonstrated a dramatic alteration in the total and CMV-specific CD8+ T-cell compartment, driving T-cells into a more terminally differentiated phenotype (Miles et al., 2007). These CMV infected infants were however shown to still mount a CD8+ T-cell response to measles vaccine equivalent to those of uninfected infants (Miles et al., 2008b). More recent work on infants in the Sukuta cohort in The Gambia has shown that EBV infected infants have lower antibody responses to measles and meningococcus polysaccharide antigens compared to uninfected children, but in contrast the CMV only infected children showed no decline in their antibody responses (Holder et al., 2010). It is also observed for reasons, which are unclear, that in sub-Saharan Africa some childhood vaccines, such as Measles, are less effective than in high-income settings (Aaby et al., 1990, Vitek et al., 1999).

This study set out to determine if antibody responses to other childhood vaccine antigens were also decreased in EBV infected infants compared to EBV naïve or CMV infected infants. Thus, the impact of infection, with the B-cell tropic EBV, in childhood on B-cell phenotypes and responses to a vaccine challenge with a pentavalent (DTP, HepB and Hib) booster vaccination given at 14-18mths of age were studied. B-cell subset disruption has been described during chronic viral infections, such as HIV, based on markers CD19, CD20, CD21

and CD38 (Moir and Fauci, 2009, Moir et al., 2008). Moir *et al.* previously looked at the distribution of B-cell subpopulations in HIV infected and uninfected adults (Moir et al., 2008) using the aforementioned markers. They describe two expanded populations of B-cells in HIV viraemic individuals, firstly ‘tissue-like’ or ‘atypical’ memory B-cells (CD19+CD20+CD21–CD27–) and secondly plasmablasts (CD27+CD21–CD20–CD38+). They have demonstrated that the first of these populations represents an exhausted B-cell phenotype with reduced proliferative ability and decreased capacity to differentiate into antibody secreting cells. An expansion in these B-cell subsets could potentially reduce antibody responses to vaccination so these B-cell subsets were enumerated in the peripheral blood of both IgM– IgG– and IgM–IgG+ Gambian children.

5.2 Methods

5.2.1 Vaccination, Multiplex immunoassays and ELISAs

The vaccination in this study was administered as part of the routine schedule in The Gambia and all children enrolled in the study had received their initial primary immunisations as per the current vaccine schedule including Hep B at birth and three doses of DTP, Hep B and Hib at two, three and four months. A commercial ELISA kit was used to measure serum IgG antibodies to Hep B details of which are given in Chapter 2 section 2.8.3. The details of the methods for the Multiplex immunoassay for simultaneous quantification of serum IgG antibodies to *Bordetella pertussis*, Hib, diphtheria toxoid and tetanus toxoid are given in Chapter 2, section 2.9.

The study time line in Chapter 4 Figure 4.1 summarises the timing of vaccination, blood sampling and assays performed. The details of the study population demographics and categorisation are provided in Chapter 4 sections 4.3.1 and 4.3.2.

5.3 Results

5.3.1 B-cell subset distributions in IgM–IgG+ children compared to IgM–IgG– children

To initially investigate the impact of EBV infection on antibody responses to vaccine antigens, the distribution of B-cell subsets in IgM–IgG+ children was compared to those of IgM–IgG– children. Figure 4.2 in Chapter 4 (section 4.3.2) shows that the total (CD19+) and classical memory B-cell populations (CD19+CD27+) do not differ between IgM–IgG– and IgM–IgG+ Gambian children. To further investigate B-cell subsets in these populations, we examined the distribution of ‘tissue-like’ or ‘atypical’ memory B-cells (CD19+CD20+CD21–CD27–) and secondly plasmablasts (CD27+CD21–CD20–CD38+) in these donors. Figure 5.1A demonstrates that there was no difference in B-cell subpopulations between IgM–IgG– and IgM–IgG+ Gambian children expressing these markers. Three IgM+IgG–/+ children similarly had B-cells subsets that did not significantly differ from the IgM–IgG+ children (Fig. 5.1A).

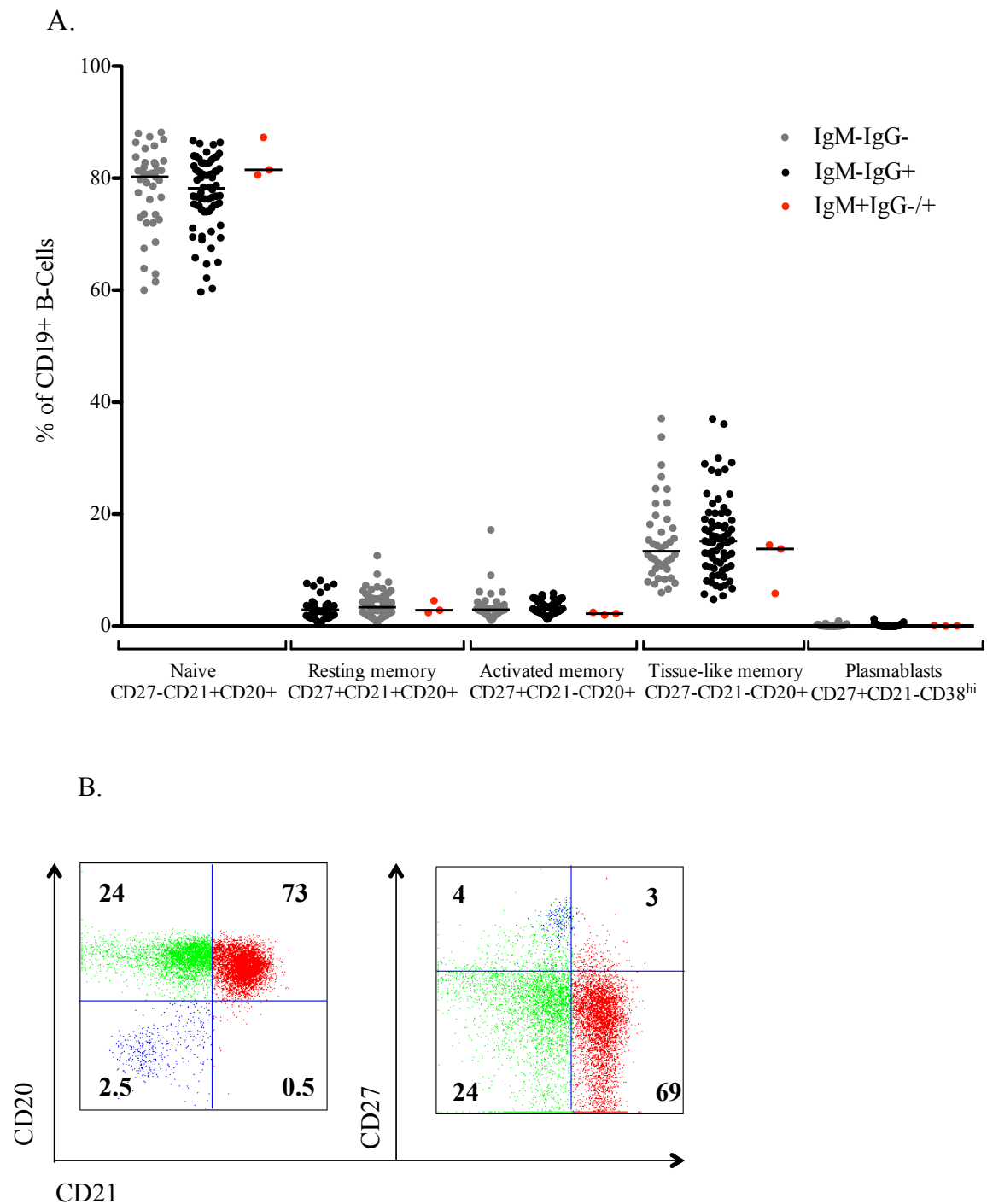


Figure 5-1 B-cell subsets in of IgM-IgG-, IgM-IgG+ and IgM+IgG-/± children.

A. Frequency of B-cell subpopulations in the blood of IgM-IgG- Gambian children (n=40) compared to IgM-IgG+ children (n=70) and IgM+IgG-/± children (n=3). B. Illustration of flow cytometry staining of B-cell subpopulations from a representative Gambian child gated on CD19+ B-cells. Colour coded gating: green (CD20+CD21-) activated (CD27+) and tissue-like (CD27-) memory B-cells; red (CD20+CD21+) resting memory (CD27+) and naïve (CD27-) B-cells; and blue (CD20-CD21-CD27+) plasmablasts. Populations are given as a percentage of total CD19+B-cells in bold.

5.3.2 Antibody responses to pentavalent *Bordetella pertussis*, Diphtheria, Tetanus, *Haemophilus influenzae B* and Hepatitis B vaccination in IgM–IgG+ children compared to IgM–IgG– children

Children aged nine months from the same study site in The Gambia who are infected with EBV have been shown to have lower antibody responses to measles and meningococcus polysaccharide antigens when infected with EBV compared to EBV uninfected children (Holder et al., 2010). To determine whether this phenomenon also occurred in response to other vaccine antigens, antibody responses in terms of titre were measured to DTP, Hib and Hep B following a pentavalent booster vaccination given at 12-14mths of age, and compared between IgM–IgG+ and IgM–IgG– children where plasma samples were available (n=101). Figures 5.2 and 5.3 show that there were no significant differences in the final boosted antibody levels nor fold changes overtime between IgM–IgG–, IgM–IgG+ and IgM+IgG–/+ Gambian children for *Bordetella pertussis* (Ptx, FHA, Fimbriae and Prn) (Figure 5.2), Diphtheria toxin, Tetanus toxoid, *Hib* and Hep B antibody levels (Figure 5.3). P values were all non-significant > 0.05. (P values calculated using Dunn’s Multiple Comparison Test (one way analysis of variance [ANOVA])

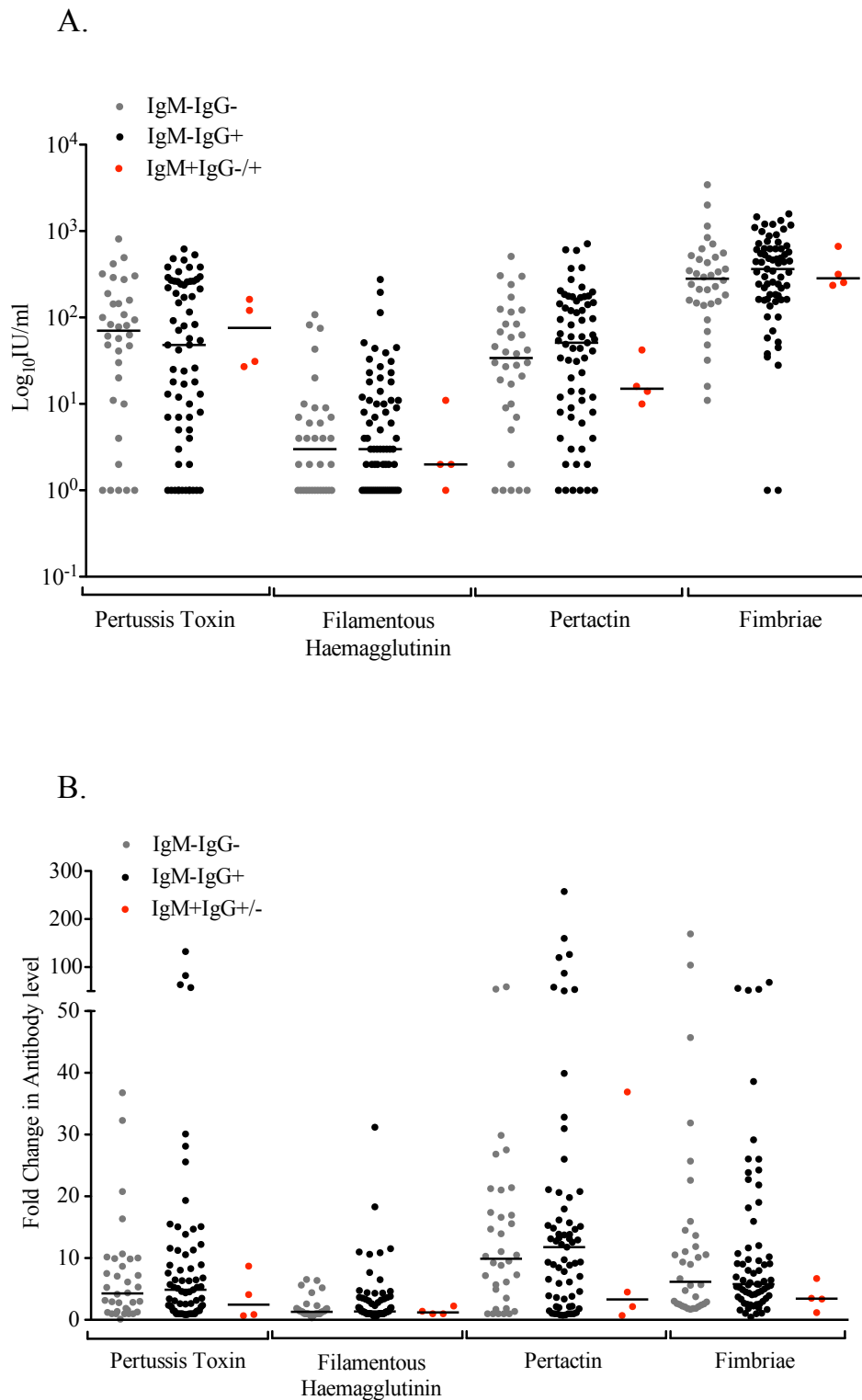


Figure 5-2 *Bordetella pertussis* antibody levels

A. One-week post vaccination and B. Fold change in antibody level post vaccination in EBV IgM–IgG– (grey circles), EBV IgM–IgG+ (black circles) and EBV IgM+IgG–/+ (red circles) Gambian children.

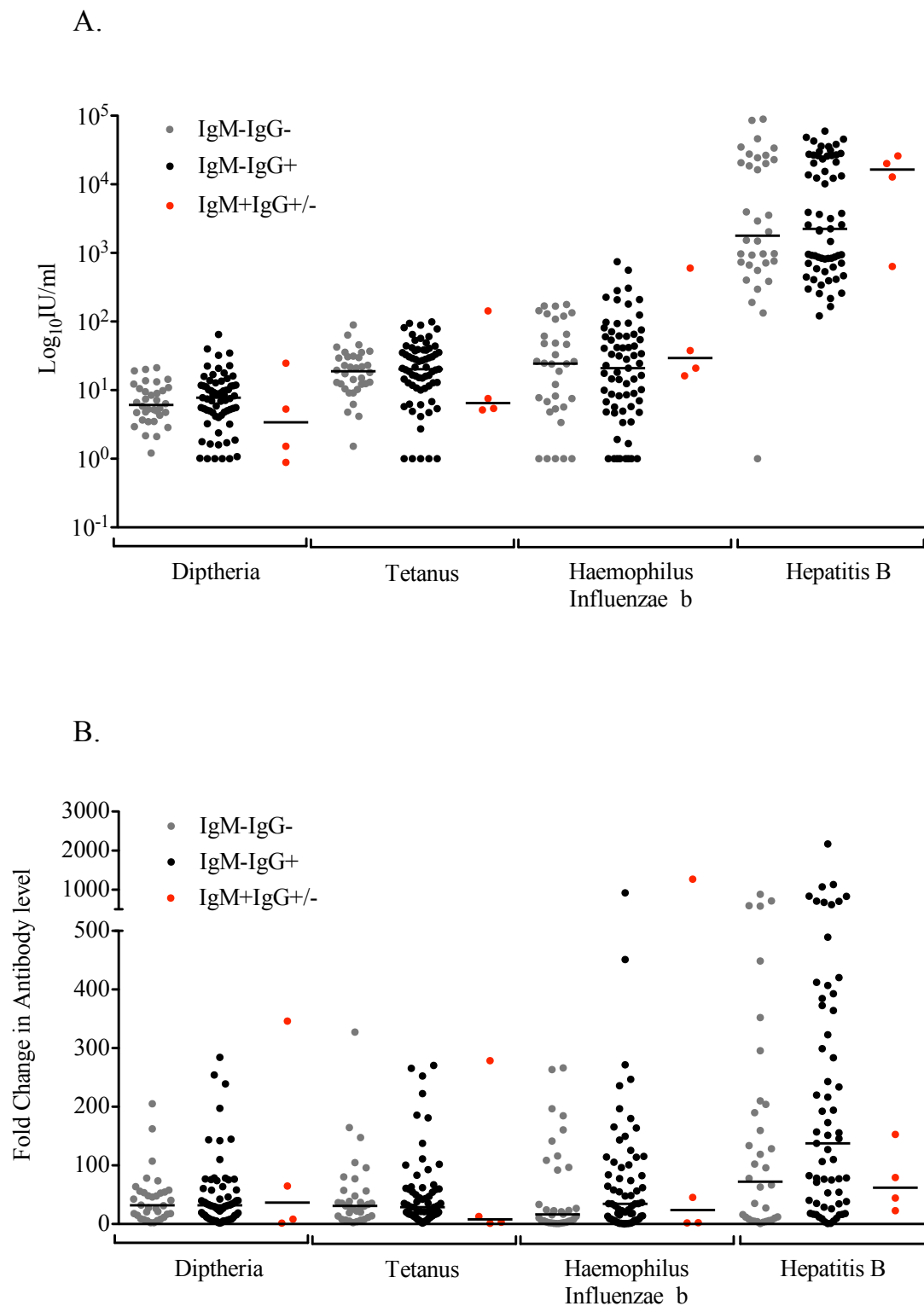


Figure 5-3 Diphtheria, tetanus, *Haemophilus influenzae b* and Hepatitis B antibody levels
A. one-week post vaccination and B. Fold change in antibody level post vaccination in EBV IgM-IgG- (grey circles), EBV IgM-IgG+ (black circles) and EBV IgM+IgG-/+ (red circles) Gambian children.

A potential confounder here is CMV status. Table 5.1 gives the breakdown of EBV and CMV status of children for who paired pre- and post vaccination antibody levels were measured (n=101). There was no significant difference in post vaccination antibody levels for *Bordetella pertussis*, DTP, Hib or Hep B between the following four groups, CMV–EBV– (n=7), CMV+EBV– (n=27), CMV–EBV+ (n=9), CMV+EBV+ (n=58) (P values calculated using Dunn’s Multiple Comparison Test (one way analysis of variance [ANOVA]) (Figure 5.5)

Table 5-1 *Two-way table illustrating EBV and CMV status of Gambian children tested for vaccine antibody responses*

	CMV infected	CMV uninfected	Total
EBV infected	58 (57%)	9 (9%)	67 (66%)
EBV uninfected	27 (27%)	7 (7%)	34 (34%)
Total	85 (84%)	16 (16%)	101

Number of children stated, percentage of total population given in brackets.

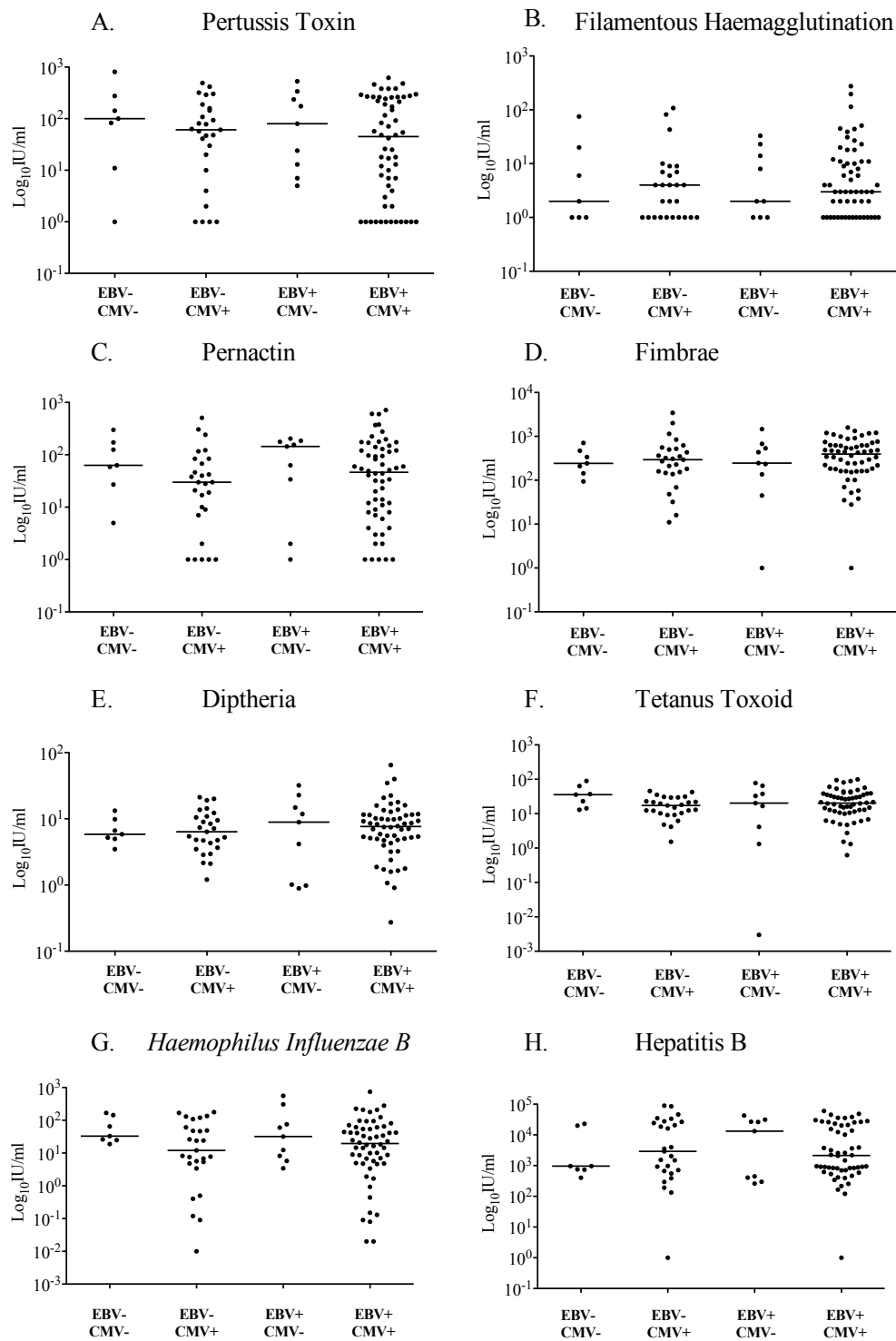


Figure 5-4 Boosted antibody levels displayed according to EBV and CMV status. Pertussis antigens: Pertussis toxin (A), Filamentous Haemagglutination (B), Pertactin (C), Fimbriae (D), Diphtheria toxoid (E), Tetanus toxoid (F), Haemophilus influenza B (G) and Hepatitis B (H) antibody levels one week post pentavalent (DTP, Hib and Hep B) vaccination booster displayed according to EBV and CMV infection status, from left to right CMV- EBV-, CMV+ EBV-, CMV- EBV+, EBV+ CMV+.

5.4 Discussion

In this chapter B-cell subsets in Gambian children aged 12-14 months known to be EBV anti-VCA IgM-IgG⁺ were explored and compared to those of EBV VCA anti IgM-IgG⁻ Gambian children. These data show that there is no significant alteration in peripheral blood B-cell phenotypes, and furthermore, no apparent defect in functioning of memory B-cells in producing a rapid antibody response to a pentavalent vaccination booster (DTP, Hib and Hep B).

Classical memory B-cell (CD27⁺) populations were not found to be expanded in the IgM-IgG⁺ children compared to those that were IgM-IgG⁻. Furthermore although numbers were small, the frequency of this population in IgM+IgG^{-/+} children did not significantly differ either. As EBV is a gamma herpesvirus that resides in the B-cell compartment specifically in memory B-cells one might have expected to see an expansion in this B-cell population (Hislop et al., 2007b). Indeed, healthy EBV seropositive adults are known to have 10 - 100 in every 10⁶ B-cells infected with EBV (Khan et al., 1996). The lack of expansion of the B-cell compartments in these Gambian children may be a reflection of the form of EBV infection occurring with the virus being in a latent rather than a lytic proliferative state (Silins et al., 2001) as discussed previously in Chapter 4.

Gambian children did appear to have an expansion of 'tissue-like' or 'atypical' memory B-cells' (CD19⁺CD20⁺CD21⁻CD27⁻), a population previously described in the lymphoid tissue of healthy individuals (Ehrhardt et al., 2005) and more recently noted to be expanded in the peripheral blood of HIV viraemic individuals (Moir et al., 2008). This B-cell subset has been shown to undergo isotype class switching and somatic hypermutation. However these

cells proliferate less well in response to BCR-crosslinking with or without CD40L and the Toll-like receptor (TLR9) agonist CpG and have a reduced ability to differentiate into antibody secreting cells compared to naïve (CD27⁻CD21⁺) or classical memory B-cells (CD19⁺CD27⁺). In contrast to the literature on adult B-cell subsets there was a considerably greater proportion of ‘tissue-like’ or ‘atypical’ memory B-cells in EBV infected Gambian children in the total B-cell population, median 13.40% compared to 1.4% previously found in US adults (Weiss et al., 2009). The expansion of ‘tissue-like’ or ‘atypical’ memory B-cells seen in these Gambian children was also found to be the case in Mali in West Africa with adults and children having ‘atypical’ memory B-cell populations of 15.5% and 9.8% respectively (Weiss et al., 2009). It has been suggested that these ‘atypical’ memory B-cell populations are expanded in response to cumulative malaria exposure (Weiss et al., 2009, Nogaro et al., 2011). This would seem unlikely in our cohort as the incidence of malaria in The Gambia has declined (Ceesay et al., 2008) and only one case of *P.falciparum* malaria was detected during the six-month recruitment period among the 120 children. As this B-cell subset was also identified in HIV infected individuals this could have been a potential confounder. A survey in 2000-2001 of pregnant women in Serekunda, an urban settlement 10km from the Sukuta study site, estimated the prevalence of HIV to be 1.0% (CI: 0.9-2.4%), which would make HIV infection unlikely to be causing an elevation in the levels of these circulating B-cells in the peripheral blood of these Gambian children. Similarly nutritional status is unlikely to explain the expansion of this B-cell subset as in this study population children were excluded if they were underweight. Further work to assess the functional significance and explore reasons behind the expansion of this ‘atypical’ B-cell subset in African populations needs to be conducted, considering the role of other possible recurrent infections and other factors such as nutritional status.

The lack of impairment in antibody response to the pentavalent booster vaccination are in contrast to the findings of Holder *et al.* who previously showed that EBV infected children recruited from the same study site, Sukuta, had reduced responses to meningococcal polysaccharide and measles vaccine compared to uninfected infants (Holder et al., 2010). A number of possibilities could account for this difference. The children in this study were three to six months older than those in the Holder study, thus their findings may relate to the relative immaturity of the immune system and not be a long-lasting effect. Indeed Holder *et al.* did note a reduction in the differences detected when their children reached 11 months of age. The effect could also be vaccine specific as diphtheria and tetanus are toxoid vaccines, pertussis is a whole bacterial cell killed vaccine, while measles a live attenuated vaccine. Furthermore, *Haemophilus influenza* type b and Meningococcus A and C despite both being polysaccharide vaccines, the later is a conjugate vaccine whereas the former is unconjugated. Further analysis of the antibody responses to Measles and Meningococcus A and C, exploring the longevity of the alteration identified by Holder et al. would be a useful addition to the literature. Lastly, due to the increasing age of the children in this study by default a greater proportion were CMV infected and Holder *et al.* found that CMV co-infection reversed the effects of EBV infection and elevated antibody responses to levels similar to infants that were EBV and CMV uninfected. However in an analysis of these data according to CMV and EBV status no such differences were seen, with the caveat of small sample sizes for the CMV uninfected groups. Similarly to Holder *et al.* only 10% of the children were in the EBV infected and CMV naïve (CMV–EBV+) category.

These data show no evidence that the phenotype of B-cells is altered as a result of EBV infection. Likewise the ability of B-cells to produce an antibody response to vaccination was

not affected. This is an encouraging result as it would be of concern if this ubiquitous viral infection had a significant impact on common vaccinations given in childhood. It would be of interest to determine which B-cell subset EBV is residing in in these Gambian children in view of this lack of expansion of memory B-cells.

CHAPTER 6 : CONCLUSIONS AND FUTURE DIRECTIONS

Although EBV was discovered some 50 years ago, we still do not fully understand its pathogenesis. This ubiquitous dynamic virus has the ability to silently infect some human hosts causing little or no apparent clinical disease at the time of initial infection and remain in a dormant state. At the other end of the spectrum are those individuals in whom the initial acute infection causes a picture of severe clinical disease with significant symptomatology known as AIM. The likelihood of a symptomatic clinical presentation alters with the age of primary infection: AIM is extremely rare in children, with most cases described in adolescence or adulthood. Why this is the case remains to be elucidated. It could be due to an alteration in the form that the virus takes in early EBV infection (dominance of lytic or latent infection) or the host response, but is likely to be multifactorial.

In West African children, EBV infections occur at a much earlier age than in developing countries, and by three years of age most children are EBV seropositive. Aside from recent modelling of the infection in Kenyan children, demonstrating that those infected at an earlier age have a higher viral load set-point in infancy (Piriou et al., 2012), little is known about the implications of early EBV infection on the immune system. Indirectly, EBV-related tumours are known to be prevalent in this population group, the classical example being eBL. Therefore, development of a vaccine against EBV to prevent establishment of a latent infection in early childhood, or perhaps more realistically alter the virus host balance in favour of the host upon infection, would be of value in reducing the incidence of EBV-related tumours in countries often lacking adequate facilities to provide sufficient treatment of them.

Studying the immune response to EBV in a setting where infection is highly prevalent at a young age is of importance in informing this process.

The other well-recognised co-factor involved in the pathogenesis of eBL is *P. falciparum* malaria infection. Chapter 3 of this thesis has built on previous work conducted in The Gambia exploring the interaction between these two pathogens and the host immune response in children. The previous findings of Whittle *et al.* some 30 years ago initially established that there was a loss of EBV-specific immune responses in children with acute *P. falciparum* infection. In this thesis both the original assays described by Whittle *et al.* were used in addition to the more contemporary techniques of peptide-MHC class I tetramer staining and IFN- γ ELISPOT analysis to directly identify EBV-specific CD8⁺ T-cells. Both of these failed to show a reduction in EBV-specific immune responses during acute *P. falciparum* infection in Gambian children. This present study was conducted on the background of a significant and well-documented decline in the incidence of malaria in The Gambia (Ceesay *et al.*, 2008, Ceesay *et al.*, 2010). This supports the hypothesis that it is not purely a single episode of acute malaria that alters the host virus balance in EBV infection, but rather the effect of cumulative malarial episodes. The work by Moormann *et al.*, who studied two cohorts with differential malaria exposure in the Kisumu and Nandi areas of Western Kenya, is relevant here. They noted higher EBV genome loads in children, one to four years of age, living in areas holoendemic for malaria compared to those only sporadically exposed (Moormann *et al.*, 2005). This is consistent with this data and supports the idea that chronic repeated exposure to malaria throughout a child's early years may be required to alter the EBV-host balance. This is yet to be directly confirmed and would require a longitudinal study, comparing immune responses to EBV at the time of first and subsequent acute malaria infections.

These findings do have more general implications, supporting the notion that the decline in malaria seen in some parts of sub-Saharan Africa has wider implications than a reduction in morbidity and mortality from malaria alone. Indeed, studies have shown changes in the rates of invasive bacterial disease such as non-typhoidal salmonella in The Gambia (Mackenzie et al., 2010, Obaro and Greenwood, 2011), and reduced all-cause mortality in children in regions where a reduction in malaria has been demonstrated (Snow and Marsh, 2010). Epidemiological studies to establish whether reduced malaria exposure also translates into a reduced incidence of eBL are needed.

To further understand the impact of EBV infection on children, antibody responses to constituent antigens of a pentavalent childhood vaccine were examined. Previous studies had suggested that nine month old children vaccinated with live attenuated measles vaccine or meningococcal polysaccharide vaccine made relatively poor antibody responses to these antigens if they were EBV infected, compared to uninfected or EBV/CMV co-infected children (Holder et al., 2010). The clear conclusion from the current study is that antibody responses to a booster dose of DTP, HepB and Hib vaccination are not altered by the presence of EBV. Why antibody responses to measles and meningococcal vaccines were reduced in the previous study in EBV infected infants is unclear, but encouragingly for children, these findings do not extend to this older age group immunised with DTP, HepB and Hib. One may speculate that the effects of EBV infection on vaccine responses are less apparent in an older age group. A way to test this would be to find children who had missed their measles and meningococcal vaccinations at the time of administration of the pentavalent booster, immunise these children with measles and meningococcal vaccines and follow responses to these two antigens in parallel with the others.

Primary EBV infections that occur sub clinically or silently are difficult to identify and therefore difficult to study. Thus, the majority of the EBV literature to date focuses on the immune response during acute symptomatic infection, AIM, or persistent infection in patients in whom there is no history of AIM. What causes someone to develop AIM, as opposed to silently seroconvert, is not yet known. In Chapter 4 we were able to identify two groups of recently EBV infected children: one group who showed serological evidence of recent infection (including the production of EBV-specific IgM antibodies), while the other were children likely to have been infected within the previous six months, based on epidemiological (Holder et al., 2010) and viral load data. A significant difference was seen in the immune responses in most of these Gambian children, when compared to those described in Caucasian donors, with or without a history of AIM: the lack of a dominance of lytic responses, with latent responses that were of a similar magnitude to lytic responses. This should be interpreted with caution, as epitopes used in this study have been defined based on work with type 1 EBV strains in Caucasians. However, while the Gambian strains of EBV in our donors were not sequenced, Njie *et al.* described previously that 90% of Gambian donors they studied carried type 1 EBV (Njie et al., 2009). This indicates that the use of peptides derived from type 1 EBV is a reasonable choice. In addition, there is extensive homology between the two subtypes and cross-reactive responses have been recognised (Bornkamm et al., 1980, Sample et al., 1990, Zimmer et al., 1986, Brooks et al., 2000). It would be prudent to undertake further sequencing of local circulating EBV strains. Designing peptide libraries to these local strains for screening responses would increase the validity of these findings. This lack of lytic dominance in Gambian children's responses contrasts with those detected in AIM, where they are predominantly lytic. During AIM, significant EBV loads are shed from

the oropharynx and this persists for sometime. Exploring EBV-specific responses in Gambian children with latent epitope-specific class I tetramers, such as B*0801 restricted FLR or QAK, would enable a direct comparison of the number of specific CD8⁺ T-cells to lytic and latent epitopes. One would predict, based on the current findings, that these would be of a similar order to the lytic EBV-specific populations detected. Furthermore, it would be of interest to examine tonsillar tissue or secretions from the oropharynx of children undergoing asymptomatic primary EBV infections using RT-PCR to determine if the degree of viral shedding differs from that seen in AIM, where the level of viral shedding has been noted to be high and persistent (Balfour et al., 2005, Hislop et al., 2005).

It has previously been shown that blocking of full virus replication with acyclovir (ACV) does not affect the clinical course of AIM once the disease is underway, although it reduces viral shedding in the oropharynx (Tynell et al., 1996). This, however, is not surprising if the initial phase of virus replication in B-cells has passed by the time a patient presents with symptoms of AIM. If ACV could be administered sufficiently early, it could potentially prevent the massive activation/expansion of lytic antigen-specific CD8⁺ T-cells by reducing EBV replication and lytic antigen load. The recombinant soluble gp350 EBV vaccine has been shown to prevent AIM (reduced the rate by 78% in young adults) but not to prevent EBV infection. The postulated mechanism behind this is that the induced neutralising antibodies reduce the initial phase of viral replication (Sokal et al., 2007). This has been explored in the similar rhesus Lymphocryptovirus (LCV) animal model, a naturally endemic virus in Rhesus monkeys (Moghaddam et al., 1997). High levels of antibody to gp350 were associated with better protection against viral challenge (Sashihara et al., 2011). This model could be used to

further explore the balance of lytic and latent cellular responses during primary EBV infection (Rivailler et al., 2002).

Another question, which arises from this thesis, is whether infant human B-cells are less permissive for EBV replication than adults B-cells, and whether this is why EBV-specific responses to latent proteins are elevated in childhood infection, but not in adults. This is an interesting possibility as it is known that EBV-transformed cord blood B-cell lines *in vitro* are predominantly latent, whereas adult B-cell lines usually have between 1-3 % of cells in lytic cycle (Alan Rickinson, personal communication).

The EBV genome load in recently infected Gambian children was as high as those found in patients suffering with AIM, despite these children being clinically asymptomatic. This corroborates the findings of Njie *et al.* where genome loads in healthy control children were of a similar order. In addition they found that EBV genome loads in Gambian adults were significantly higher than UK carriers (Njie et al., 2009). What leads to both Gambian adults and children having higher viral load set-points, through alteration in the virus: host balance, is not clear. Parallels can be drawn with what has been shown in the aging Caucasian population, where EBV genome loads in the peripheral blood B-cell compartment are elevated (Snider et al., 2012, Stowe et al., 2007). EBV-specific CD8⁺ T-cells directed against latent epitopes assessed in the elderly have been readily detected and even predominate over lytic in some instances (Khan et al., 2004, Vescovini et al., 2004), reminiscent of the equivalent frequencies of EBV-specific latent and lytic CD8⁺ T-cells observed in this study. Thus one may hypothesise that there is suboptimal EBV-specific immune control in African children or elderly populations: in Africans this may manifest as an increased susceptibility to

eBL when the co-factor of malaria is present, while in the elderly, other diseases may manifest such as EBV-associated diffuse large B-cell lymphoma of the elderly, which occurs in patients greater than 50 years with no known history of immunodeficiency or lymphoma.

In children identified as having serological evidence (IgM positive) of recent infection, it was possible to identify significant EBV-specific CD8⁺ T-cell populations, but not of the order of magnitude (in terms of absolute numbers) of EBV-specific CD8⁺ T-cells described in patients with AIM. They also lacked the total lymphocytosis classically associated with AIM patients. EBV viral genome loads in these Gambian children were substantially elevated to levels that did not significantly differ from AIM patients, and therefore symptomatic infection could not be explained by reduced viral load. One possible explanation worth exploring to explain these contrasting courses of primary infection could be differing levels of circulating inflammatory cytokines elicited. It is possible that during AIM the predominating lytic immune responses drive the production of high levels of circulating cytokines, which in turn drives the inflammatory response and results in symptomatic disease. In the asymptomatic primary infections the virus may be in its latent form and relatively protected from immunosurveillance, hence result in a less aggressive inflammatory response, leading to minimal clinical symptomology. Rare EBV infections of NK and T-cells have been described, such as EBV-associated haemophagocytic lymphohistiocytosis, where clinical manifestations are due to the release of pro-inflammatory cytokines causing dysregulation of the inflammatory response (Fox et al., 2011). This is, however, due to direct infection of NK and T-cells, rather than immunopathology due to virus-specific responses. X-linked lymphoproliferative disease (XLP) patients are vulnerable to severe primary EBV infection, often leading to lethal AIM. They have mutations in signalling lymphocytic activation

molecule (SLAM) associated protein (SAP), an adaptor that binds and recruits signalling molecules to SLAM family receptors expressed on T-cells and NK cells. The mechanism of increased susceptibility is thought to be driven by an EBV-induced overactive T/NK-cell response that is ineffective when engaging B-cells, and thus unable to clear the source of antigen leading to immune pathology (Hislop et al., 2010).

Further evidence may be required to determine whether a different form of infection is occurring in these children undergoing apparently primary asymptomatic infection, in terms of a more latent versus lytic infection (Silins et al., 2001). In this case, examining these asymptomatic patients for virus loads shed from the oropharynx, indicative of lytic virus replication, could be undertaken. Clearly further studies are required as to how the immune response differs in these children compared to AIM patients. Most of the primary infection donors made responses to EBV-epitopes, which were smaller than those seen to epitopes in AIM patients. However at least one donor showed a clear expansion of EBV-specific T-cells in response to a lytic epitope which, when measured in terms of percentage tetramer binding CD8⁺ T-cells, was comparable to what is seen in AIM patients. Crucially this and other donors did not show a lymphocytosis when compared to age matched controls. At face value, this may suggest EBV infection could elicit strong CD8⁺ T-cell responses, but the repertoire stimulated or expansion of responses in these donors are highly focused in contrast to AIM and thus a lymphocytosis is not observed in these children. Examining co-resident EBV-specific responses in such asymptomatic donors would go some way to resolve this issue.

Could the difference in the lytic:latent balance of EBV-specific T-cell responses and the presence or absence of a lymphocytosis in Gambian children compared with adolescent AIM

donors be an age related phenomenon? Infection during adolescence may result in a greater propensity for a lytic-biased response, as well as a significant lymphocytosis, with AIM being at the extreme end of a clinical spectrum. Indeed, recent data from the Balfour *et al.* study found that viral load and CD8+ T-cell percentage correlated with a severity of illness index (Balfour et al., 2013). Exploration of immune responses in asymptomatic primary Caucasian adolescent donors (with a similar approach to that taken in the current study) may shed further light on this finding. However, if there were equivalent lytic and latent responses and an absence of a lymphocytosis seen in both asymptomatic adolescent seroconverters and Gambian children, this would suggest factors other than age result in a lytic-biased response and therefore symptomatic infection/AIM.

Overall it is clear that there is still much we do not understand about what dictates the clinical phenotype of primary EBV infection. A greater understanding of the immunopathology and consequences of infection will provide further targets for immune therapy and potential vaccine candidates. This body of work furthers our understanding of the impact of EBV infection in childhood in West Africa, an area where eBL is well recognised. It adds to the body of literature surrounding EBV and malaria co-infection. Age related differences in primary infection are highlighted, and important insights provided regarding the cellular immune response to EBV infection in otherwise healthy Gambian children.

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APPENDIX I. INTRACELLULAR CYTOKINE STAINING (ICS)

Co-expression of four cytokines (IFN- γ , IL-2, TNF- α and MIP1 β) and capacity to degranulate (surface expression of CD107a) following stimulation with overlapping peptide pools spanning EBV lytic protein, BZLF1 and EBV latent protein, EBNA3A was assessed.

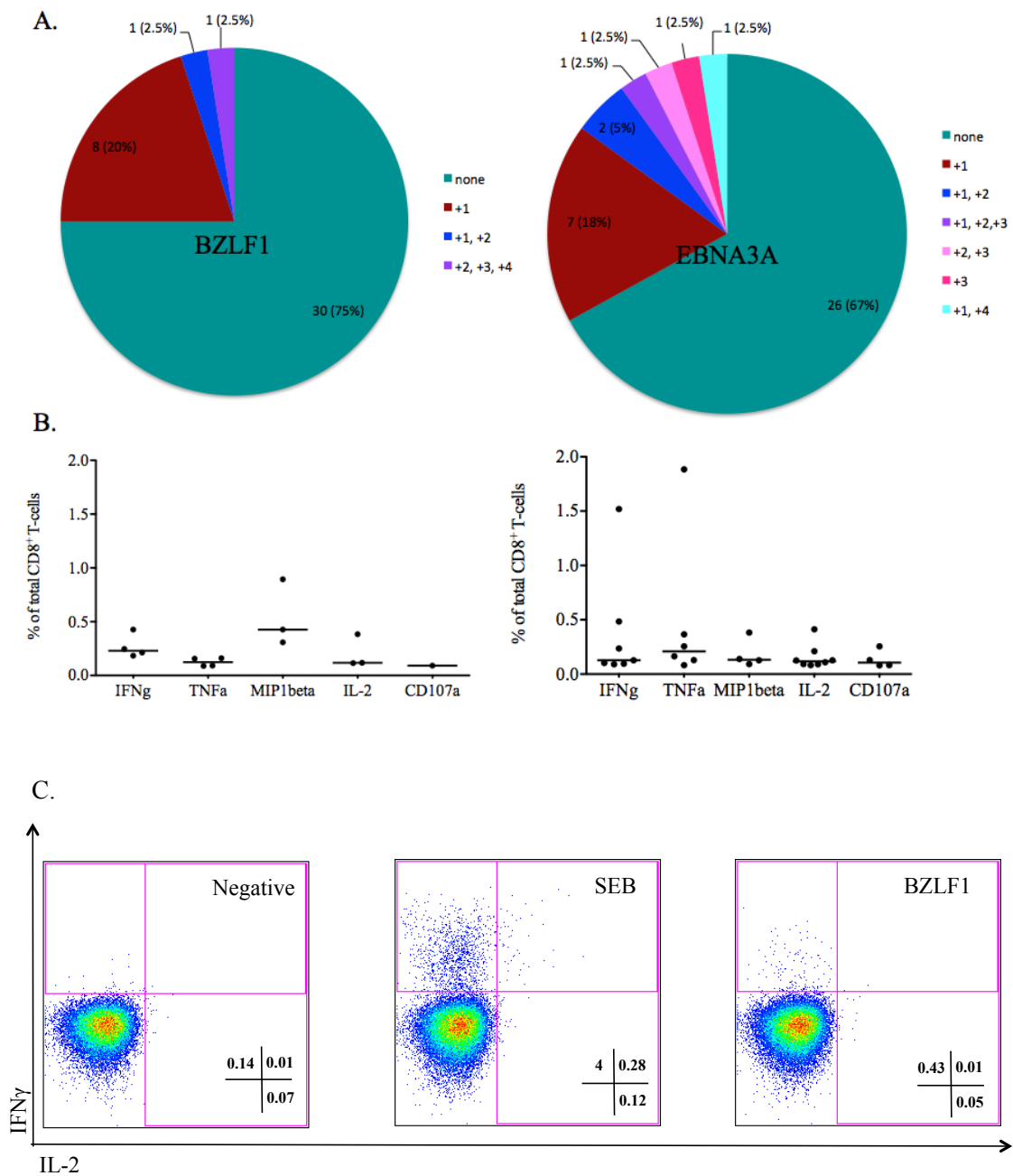
Polyfunctional analysis of CD8+ and CD4+ EBV-specific T-cells.

So called ‘polyfunctional cells’ based on their ability to produce multiple cytokines and possession of cytotoxic capacity have become increasingly thought to provide correlates of immune protection against pathogenic viruses. Some recent work performed on healthy Hong Kong Chinese adult long-term EBV carriers demonstrated that IFN- γ producing EBV-specific T-cells accounted for only 40-50% of the total EBV-specific CD8+ and CD4+ T-cell populations, therefore half the EBV-specific T-cells did not produce IFN- γ (Ning et al., 2011). This suggests that by focusing on IFN- γ alone many cell responses may be missed. Ning et al. also studied two children with PTLD showing that they produced less IFN- γ and IL-2, but more TNF- α with retained ability to degranulate compared to adult EBV carriers. This could be an age-specific effect or a consequence of immunosuppression, as these children were on continuous immunosuppressive therapy (Ning et al., 2011).

In this section IgM⁻IgG⁺ donors were assessed for the co-expression of cytokines (IFN- γ , TNF- α , MIP1 β and IL-2) and the surface degranulation marker, CD107a, in both CD8+ and CD4+ T-cells by stimulating with overlapping peptide pools spanning BZLF1 lytic and EBNA3A latent proteins (n=40 and 39 donors respectively). The ICS assays were performed only on children at the baseline first time point. The majority of EBV-specific CD8+ T-cells

in IgM[−]IgG⁺ Gambian children were not polyfunctional. Indeed, the majority of CD8⁺ T-cells failed to produce any cytokines in response to stimulation with BZLF1 lytic (30 donors) or EBNA3A latent (26 donors) peptide pools (Figure 1.0 A and B). The few responders that were detected tended to have monofunctional populations (n=8 BZLF1, n=7 EBNA3A) with only two (20%) and six (46%) of responding donors producing polyfunctional responses to stimulation with BZLF1 and EBNA3A peptide pools respectively (Figure 1.0 A). Of the monofunctional responders three different donors for BZLF1 (38%) and EBNA3A (43%) produced IFN- γ . There were only four (21%) donors that produced responses to both BZLF1 and EBNA3A peptide pools. The rest responded either to the BZLF1 pool (6, 32%,) or the EBNA3A pool (9, 47%).

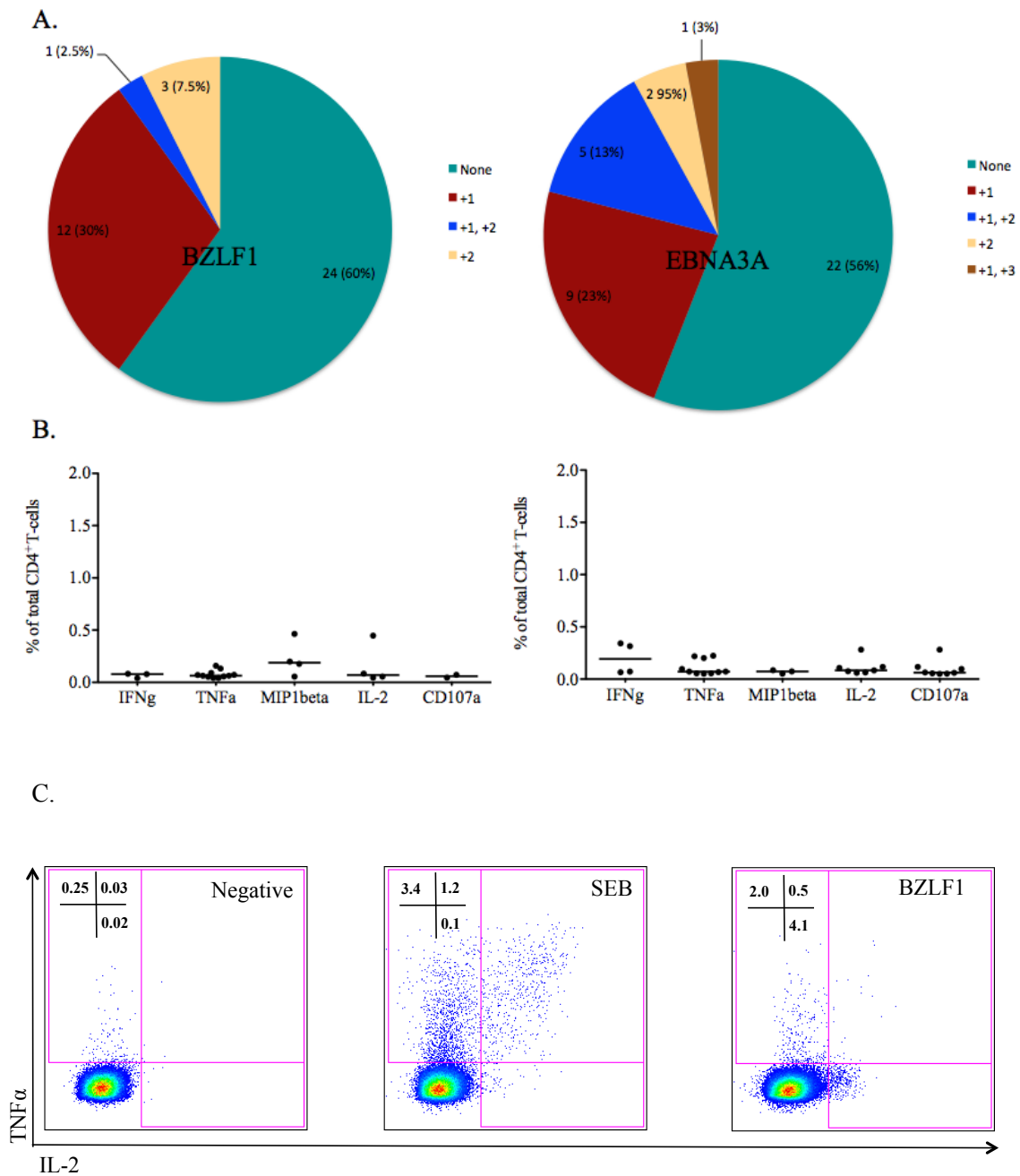
As there were so few responders it did not make sense to analyse the polyfunctionality of the cells (number of cytokines individual cells were producing (mono-, bi- or triple- functionality etc.), as a proportion of the overall response using averages as the overwhelming majority of responses were zero. Therefore SPICE software was not used for further analysis. Figure 1.2 gives the proportion of donors with cells that were monofunctional, bi-functional or triple functional or a combination of the aforementioned as indicated in the Figure legend.



Polyfunctional cell populations: +1 = monofunctional cells, +1, +2 = mono and dual-functional cells, +2 = dual functional cells, +1 +3 mono and triple functional cells

Figure 1 A. Breakdown of donor CD8+ T-cell responses when stimulated with BZLF1 lytic (left, n=40) and EBNA3A latent (right, n=39) protein overlapping peptides pools. Displayed are absolute numbers (percentages in brackets) of donors with either no response, monofunctional or polyfunctional responses. B. Total (mono- and polyfunctional) percentage of cytokine (IFN- γ , TNF- α , MIP1 β and IL-2) secreting CD8+ T-cells in response to BLZF1 (left) and EBNA3A (right) overlapping peptide pools and percentage of CD8+ T-cells co-expressing CD107a in addition to secreting at least one other cytokine in response to stimulation with BZLF1 (left) and EBNA3A (right) overlapping peptide pools. C. Representative donor illustrating a CD8+ T-cell IFN- γ response mounted to the BZLF1 overlapping peptide pool.

Similarly the majority of EBV-specific CD4⁺ T-cells in IgM[−]IgG⁺ Gambian children were not polyfunctional. A slightly greater number of donors had cytokine producing CD4⁺ T-cells compared to the number of donors that had detectable EBV-specific CD8⁺ T-cells responses to BZLF1 lytic protein (10 CD8⁺ responders versus 16 CD4⁺ responders) and EBNA3A latent protein (13 CD8⁺ responders versus 17 CD4⁺ responders) (Figure 1.2 A and B). The responders that were detected again tended to have monofunctional populations (n=12 BZLF1, n=9 EBNA3A) with four and eight of responding donors producing polyfunctional responses to stimulation with BZLF1 and EBNA3A peptide pools respectively (Figure 1.2 A). Of the monofunctional responders there was a greater proportion of responders producing TNF- α , (58% BZLF1, 44% EBNA3A), with only three (25%) and two (22%) donors producing IFN- γ in response to BZLF1 and EBNA3A peptide pools respectively. There were only seven (27%) donors that produced responses to both BZLF1 and EBNA3A peptide pools. The rest responded either to the BZLF1 pool (9, 35%) or the EBNA3A pool (10, 38%).



Polyfunctional cell populations: +1 = monofunctional cells, +1, +2 = mono and dual-functional cells, +2 = dual functional cells, +1 +3 mono and triple functional cells

Figure 1.2 A. Breakdown of donor CD4⁺ T-cell responses when stimulated with BZLF1 lytic (left, n=40) and EBNA3A latent (right, n=39) protein overlapping peptides pools. Displayed are absolute numbers (percentages in brackets) of donors with either no response, monofunctional or polyfunctional responses. B. Total (mono- and polyfunctional) percentage of cytokine (IFN- γ , TNF- α , MIP1 β and IL-2) secreting CD4⁺ T-cells in response to BLZF1 (left) and EBNA3A (right) overlapping peptide pools and percentage of CD4⁺ T-cells co-expressing CD107a in addition to secreting at least one other cytokine in response to stimulation with BZLF1 (left) and EBNA3A (right) overlapping peptide pools. C. Representative donor illustrating a CD4⁺ T-cell TNF- α and IL-2 response mounted to the BZLF1 overlapping peptide pool.

There were three IgM⁺IgG⁻/+ donors for which there were sufficient cell numbers to perform the ICS. Donor ID053 produced no response to either of the overlapping peptides pools, BZLF1 and EBNA3A. However, two donors both had CD8⁺ T-cells that responded to the BZLF1 lytic overlapping peptide pool and not to the EBNA3A latent pool. For donor ID061 the response was monofunctional, with cells producing either IFN- γ or MIP1 β and the second donor ID013 again produced a monofunctional MIP1 β response, but in addition to this there were cells with some dual functionality producing both IL-2 and MIP1 β . The EBV-specific CD4⁺ T-cells in these donors were similar to the responses seen for the CD8⁺ T-cells. The CD8⁺ non-responder ID053 also had no CD4⁺ T-cell response. Donors ID013 and ID061 which had CD8⁺ responses also had CD4⁺ T-cells that responded to the BZLF1 lytic overlapping peptide pool but not to the EBNA3A latent pool. For donor ID061 the response was again monofunctional, but with CD4⁺ T-cells producing either IFN- γ or TNF- α . Donor ID013 also produced monofunctional MIP1 β and IL-2 responses, with some cells showing dual functionality by producing both IL-2 and MIP1 β , similar to the CD8⁺ T-cell response. These findings are consistent with the ELISPOT reactivity, which detected an IFN- γ response in donor ID061, the donor that produced an IFN- γ by ICS.